

RESPONSE

The Examiner states in the Office Action Summary that claims numbered 1, 2, 7, 17, 45-47, 54 and 55 are pending in the application; claims numbered 1, 2, 7, 17, 45-47, and 55 are allowed; and claim number 54 is rejected.

(1) The Examiner has rejected claim number 54 under 35 U.S.C. §112, first paragraph, for failing to comply with the written description requirement. Specifically: the examiner states the specification, while being enabling for the treatment of diabetes or obesity, does not reasonably provide enablement for the treatment of other conditions mediated by the Peroxisome Proliferator-Activated Receptors (PPARs).

Applicant respectfully requests that the Examiner consider the two *review* articles enclosed with this response as being relevant to treatment of conditions mediated by the PPARs: "The PPARs: From Orphan Receptors to Drug Discovery", Willson et al., *Journal of Medicinal Chemistry*, Vol. 43, Number 4, pages 527-550 (February 24, 2000); and "Physiological and Therapeutic Roles of Peroxisome Proliferator-Activated Receptors", Berger et al., *Diabetes Technology & Therapeutics*, Volume 4, Number 2, pages 163-174 (2002). These review articles were published in 2000 and 2002, but draw the vast majority of the articles cited from 1999 and prior.

To place the claim in better condition for appeal, Applicant has amended claim number 54 to recite diseases/disorders found on page 1, lines 17-25 of the specification as originally filed.

Applicant respectfully requests reconsideration and withdrawal of the rejection under 35 U.S.C. §112, first paragraph.

In view of the above, Applicant respectfully submits all claims are in condition for allowance. *Applicant respectfully requests notification via an Advisory Action or telephone call if the Examiner deems the case is not in condition for allowance.*

Attorney Docket No. 5698.220-US
Jeppesen et al.
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The Examiner is hereby invited to contact the undersigned by telephone if there are any questions concerning this amendment or application. Applicant respectfully requests that a timely Notice of Allowance be issued in this case.

Respectfully submitted,

Date: August 5, 2004



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Perspective

The PPARs: From Orphan Receptors to Drug Discovery[†]

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Introduction

Dietary fat intake is an environmental factor that affects many aspects of human health.¹ Several lines of evidence suggest that common diseases of modern society are associated with high-fat Western diets combined with a sedentary lifestyle.² For example, diabetes, obesity, and cardiovascular disease are major causes of mortality and morbidity whose incidence tracks with the rate of industrialization in many countries.³ Conversely, it has been established that caloric restriction with low-fat diets in rodents and primates leads to increased longevity and lower incidence of metabolic and cardiovascular disorders.⁴ Interestingly not all dietary fat is bad; diets high in monounsaturated fatty acids (the Mediterranean diet) or polyunsaturated fatty acids (the Eskimo diet) appear to have cardioprotective effects.^{5,6} The epidemiology of human metabolic diseases and animal feeding studies support the proposal that caloric intake plays an important role in the regulation of lipid metabolism, insulin sensitivity, glucose homeostasis, and atherosclerosis.^{1,7} It is therefore not surprising that mammals have evolved with hormonal systems to regulate the physiological response to dietary intake of fatty acids.⁸ A family of transcription factors known as the Peroxisome Proliferator-Activated Receptors (PPARs) plays a central role in regulating the storage and catabolism of dietary fats. The PPARs were cloned less than a decade ago as orphan members of the nuclear receptor gene family that includes the receptors for the steroid, retinoid, and thyroid hormones.^{9,10} This review covers

the rapid progress in the functional analysis of these orphan receptors, research which has led to a greater understanding of the importance of fatty acids as hormones and has established the PPARs as molecular targets for the development of drugs to treat human metabolic diseases.

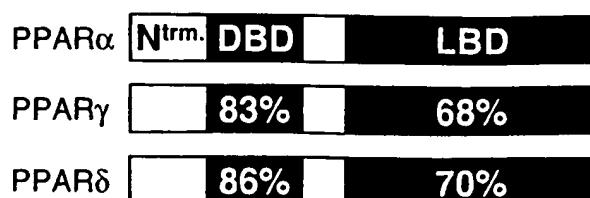
Functional Domains and Bioassays

There are three PPAR subtypes, which are the products of distinct genes and are commonly designated PPAR α [NR1C1], PPAR γ [NR1C3], and PPAR δ [NR1C2].¹¹ The PPARs possess a domain structure common to other members of the nuclear receptor gene family (Figure 1A). Sequence comparison of their DNA-binding domains (DBD) shows that they are highly conserved, while the ligand-binding domains (LBD) have a slightly lower level of conservation across the subtypes. Within the LBD certain conserved amino acids have been mapped to critical receptor functions involved in signal transduction. However, there is significant sequence variation in the residues that line the ligand-binding pocket,¹²⁻¹⁴ which is reflected in the fact that each receptor subtype is pharmacologically distinct.¹⁵ The N-terminal region of the receptor shows low sequence identity across the subtypes and is in general less well-characterized. Recent data suggests that the N-terminal domain is responsible for differences in the biological function of the subtypes.¹⁶ In addition, phosphorylation of the PPAR N-terminal domain by mitogen-activated protein kinase can affect the transcriptional activity¹⁷⁻²¹ and possibly ligand binding of the receptor.²² The PPARs form heterodimers with another nuclear receptor, the 9-cis-retinoic acid receptor (RXR) [NR2B].¹¹ The PPAR/RXR heterodimers bind to DNA sequences containing direct repeats of the

[†] Dedicated to the memory of Gordon L. Hodgson, 1945-2000.

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A



B

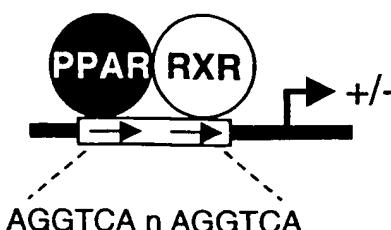


Figure 1. A. Functional domains of the PPARs: N^{term.}, N-terminus; DBD, DNA-binding domain; LBD, ligand-binding domain. Numbers represent the percent (%) identity between the human subtypes. B. PPAR/RXR heterodimer binds to a DR-1 response element in the regulatory regions of target genes. When an agonist ligand binds to the heterodimer, recruitment of coactivator proteins (not shown) leads to transcriptional activation.

hexanucleotide sequence AGGTCA separated by one nucleotide, known as DR-1 response elements (Figure 1B).²³ This sequence or minor variants have been characterized within the promoter regions of PPAR target genes such as acyl-CoA oxidase (*AOX*) and the adipocyte fatty acid-binding protein (*aP2*).²⁴

In common with other members of the nuclear receptor gene family, the PPARs are ligand-activated transcription factors. The binding of agonist ligands to the receptor results in changes in the expression level of mRNAs encoded by PPAR target genes. This process is known as 'transactivation', and cell-based assays have been developed which monitor this functional activity. Transactivation assays use cells that have been transfected with a vector expressing the receptor as well as a second vector containing a DR-1 response element and a reporter gene, which encodes an enzyme such as chloramphenicol acetyltransferase, secreted placental alkaline phosphatase (SPAP), or firefly luciferase.²⁵ Activation of the receptor by agonist ligands leads to induction of reporter enzyme expression, which can be conveniently assayed using standard colorimetric or photometric plate readers. Either PPAR or RXR ligands can induce reporter expression, indicating that the PPAR/RXR heterodimer is the functional transcription factor within cells.²³ Although the standard transactivation assay is a powerful and widely used assay, the results can be complicated by the presence of other nuclear receptors within the host cells that bind to the same DR-1 response element as the PPAR/RXR heterodimer. Modified variants of the transactivation assay exploit the observation that the LBD and DBD are autonomous domains, such that they maintain their ligand- or DNA-binding properties when expressed as chimeric proteins with other nuclear receptors or transcription factors.²⁶ Of particular note is the PPAR-GAL4 transactivation assay, which uses chimeric receptors

Table 1. Activity of PPAR Agonists in Cell-Based Transactivation Assays^a

compound	murine receptor EC ₅₀ (μM)			human receptor EC ₅₀ (μM)		
	PPAR α	PPAR γ	PPAR δ	PPAR α	PPAR γ	PPAR δ
Wy-14643	0.63	32	ia at 100	5.0	60	35
clofibrate ^b	50	~500	ia at 100	55	~500	ia at 100
fenofibrate ^b	18	250	ia at 100	30	300	ia at 100
bezafibrate	90	55	110	50	60	20
GW 9578	0.005	1.5	2.6	0.05	1.0	1.4
troglitazone	ia	0.78	ia	ia	0.55	ia
pioglitazone	ia	0.55	ia	ia	0.58	ia
rosiglitazone	ia	0.076	ia	ia	0.043	ia
KRP-297	10	0.14	7.2	0.85	0.083	9.1
JTT-501 ^b	4.3	0.089	ia	1.9	0.083	ia
SB 213068	0.93	0.10	ia	0.74	0.066	ia
GI 262570	ia	0.00035	ia	0.45	0.00034	ia
GW 1929	ia	0.013	ia	ia	0.0062	ia
GW 7845	ia	0.0012	ia	3.5	0.00071	ia
GW 0207	ia	0.14	ia	ia	0.044	ia
L-796449	7.6	0.010	0.023	0.0041	0.0052	0.0079
L-165041	ia	10	3.8	10	5.5	0.53
GW 2433	0.27	1.5	0.41	0.17	2.5	0.19

^a All data was generated using the PPAR-GAL4 transactivation assay using an SPAP reporter as described (ref 177); $\pm 20\%$, $n \geq 3$; ia = inactive at 10 μM or the concentration indicated. ^b Data is for the active metabolite.

where the PPAR LBD is fused to the DBD of the yeast transcription factor GAL4 and employs a reporter gene containing a GAL4 response element.²⁷ Since mammalian cells do not contain GAL4, only the transfected PPAR-GAL4 chimeric receptors can activate the reporter gene, effectively eliminating interference from endogenous nuclear receptors. In general, PPAR agonists show comparable potency and efficacy in assays using either the PPAR-GAL4 chimeras or the full-length receptors. The potency and selectivity of several classes of PPAR agonists in the PPAR-GAL4 assay are presented in Table 1.

Biochemical and genetic studies of the mechanism of transcription regulation have identified a series of accessory proteins that bind to nuclear receptors in a ligand-dependent manner.^{28,29} These so-called 'coactivator' proteins promote the initiation of transcription and can be loosely categorized into three classes: proteins with histone acetylase activity that remodel chromatin structure (e.g. SRC1^{30,31} and CBP/p300³²), members of the DRIP/TRAP complex that interact with the basal transcriptional machinery (e.g. PBP/TRAP220^{33,34}), and those proteins with poorly defined function (e.g. PGC1,³⁵ RIP140,^{36,37} and ARA70³⁸). Cell-free ligand sensing assays have been developed that exploit the ligand-dependent recruitment of coactivator proteins to the receptor. The coactivator-dependent receptor ligand assay (CARLA) detects the binding of a ³⁵S-labeled fragment of SRC1 to the PPAR LBD on an acrylamide gel.³⁹ Recently, the use of fluorescence resonance energy transfer has allowed the development of homogeneous CARLA assays.⁴⁰ These assays can be used as high-throughput cell-free screens for the identification of new PPAR ligands and for the characterization of coactivator recruitment to the receptor.

Several radioligands are now available for use in conventional competition binding assays: [³H]GW 2331 for PPAR α ,⁴¹ [³H]rosiglitazone (BRL49653),²⁷ [³H]AD-5075,⁴² and [¹²⁵I]SB-236636⁴³ for PPAR γ ; and [³H]GW 2433⁴⁴ and [³H]L-783483⁴⁵ for PPAR δ . Each of these radioligands is reported to show specific binding to the

corresponding PPAR subtype. Initial PPAR binding assays used gel filtration to separate the bound radioligand from the free ligand.^{27,41} However this technique is not ideal for high-throughput screening. The use of scintillation proximity assay (SPA) technology has greatly increased the throughput of PPAR competition binding assays.^{46,47} SPA beads emit light when held in close proximity to a suitable radionuclide (e.g. ³H or ¹²⁵I). If the receptor is attached to the SPA bead, binding of a radioligand to the PPAR LBD leads to a readily detectable signal. Displacement of the radioligand by a test compound leaves the free radioligand in solution, where it can no longer promote emission by the SPA bead. This technology removes the need to separate free radioligand from the bound ligand, greatly simplifying automated high-throughput screening. The homogeneous format of the SPA binding assay allows determination of equilibrium binding affinities and also permits the use of relatively low-affinity radioligands.⁴⁶

The biology of the PPARs has been driven, in large part, by the availability of potent and selective ligands for the receptors.⁴⁸ Through the use of binding and functional assays, several groups have reported the identification and optimization of PPAR ligands for each of the three subtypes (Table 1). These chemical tools have been used in a "reverse endocrinology" approach to uncover the role of the PPARs in human physiology and disease processes.⁴⁹

PPAR α

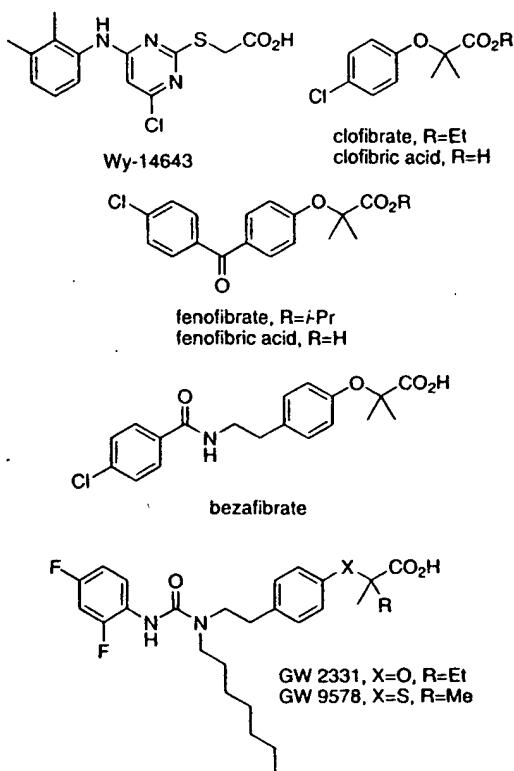
PPAR α was originally cloned from a mouse liver cDNA library⁹ and has since been cloned from frogs,⁵⁰ rats,²⁶ guinea pigs,⁵¹ and humans.^{52,53} Although the DBDs are identical across a variety of species, the LBDs exhibit lower homology, which may reflect evolutionary adaptation to different dietary ligands. Comparison of human PPAR α to the murine PPAR α shows an 85% identity at the nucleotide level and 91% identity at the amino acid level. The human PPAR α gene has been mapped to chromosome 22q12-q13.1 by somatic cell hybridization and linkage analysis.⁵² Analysis of PPAR α tissue distribution in rodents and humans revealed high levels of expression in metabolically active tissues, such as liver, heart, kidney, and muscle.^{54,55} PPAR α mRNA levels are under diurnal control of endogenous glucocorticoids in at least some of these tissues.^{56,57}

PPAR α is activated by a diverse range of compounds, such as fibrates and plasticizers, which cause the proliferation of peroxisomes and hepatomegaly in rodents.⁹ This phenomenon has not been observed in nonrodent species, including humans, even though compounds that cause peroxisome proliferation activate both human and murine PPAR α in cell-based transactivation assays.⁵⁸ Thus, although administration of peroxisome proliferators to rodents results in hepatomegaly and hepatocellular proliferation accompanied by suppression of apoptosis,⁵⁹ years of clinical experience with fibrates in humans has not led to evidence of peroxisome proliferation, as determined by morphological examination of liver biopsies,^{60,61} or to an increased incidence of liver cancer.⁶²⁻⁶⁴ The molecular basis of this species-specific response may be due to differences in the function of PPAR α in rodents and humans. For example, there are differences in the hepatic expression

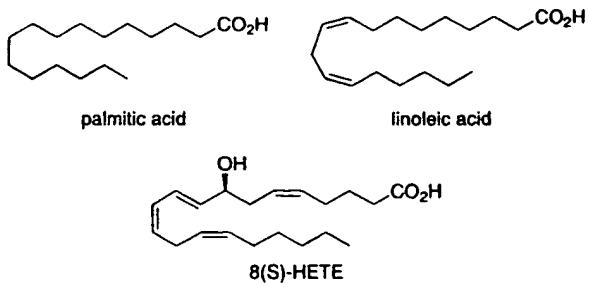
of PPAR α across species. The wild-type PPAR α is expressed in rodent livers at 10 times higher levels than in human livers.⁶⁵ In addition, a splice-variant of human PPAR α , which represents 20–50% of the total PPAR α mRNA in the liver, has been identified in which exon 6 is deleted.^{65,66} The truncated human receptor lacks a LBD and was also unable to bind to a DR-1 response element. Although the truncated isoform inhibited the activity of wild-type receptor under certain conditions, immunolocalization showed that it was not normally present in the nucleus of cells.⁶⁶ A homologous truncated PPAR α transcript could not be detected in rodent liver. Differences in the expression levels of PPAR α and its isoforms may be only one factor affecting the species-specific response to peroxisome proliferators. It has been noted that the DR-1 response elements of key peroxisomal genes such as *AOX* are not conserved between rodents and humans.⁶⁷ Therefore, the physiological role of PPAR α as a regulator of peroxisome function appears to be restricted to rodents, and the common designation of this receptor (and the other PPAR subtypes) does not reflect its biological function in humans.

Synthetic Ligands. The hypolipidemic fibrate drugs are an important class of PPAR α ligands. Wy-14643 was identified as a micromolar activator of murine PPAR α using a cell-based transactivation assay.⁹ This drug as well as clofibrate, fenofibrate, and bezafibrate were developed as hypolipidemic agents through optimization of their lipid-lowering activity in rodents before the discovery of the PPARs. Table 1 shows the potencies of several fibrate drugs on the human and murine PPARs. Clofibrate acid and fenofibrate acid, the active metabolites of clofibrate and fenofibrate, are dual activators of PPAR α and PPAR γ , with ~10-fold selectivity for PPAR α , while bezafibrate activates all three PPAR subtypes at comparable doses. All of these compounds require high micromolar concentrations to activate human PPAR α , which may explain why high doses (300–1200 mg/day) are required for their clinical activity.⁶⁸ Attempts to identify more potent compounds led to the synthesis of a series of ureidofibrates that were active at lower doses in rodent models of hyperlipidemia.⁶⁹ One of these ureidofibrates, GW 2331, was shown to bind to both PPAR α and PPAR γ with nanomolar affinity.⁴¹ Chemical libraries of ureidofibrates have been synthesized on solid-phase,⁷⁰ which has enabled this class of ligands to be rapidly optimized for PPAR activity on the human receptors.⁴⁴ The closely related ureidothioisobutyric acid GW 9578 has recently been reported as a potent and subtype-selective PPAR α agonist with improved lipid-lowering activity compared to fenofibrate.⁷¹

Natural Ligands. Several groups have implicated fatty acids as natural ligands for PPAR α . The Gustafsson laboratory was the first to demonstrate that a range of saturated and unsaturated fatty acids could activate PPAR α .²⁶ A search for natural PPAR α ligands in human serum identified palmitic acid, oleic acid, linoleic acid, and arachidonic acid as endogenous activators of rat PPAR α .⁷² Direct binding of fatty acids to PPAR α has been demonstrated using competition binding assays^{14,41} and cell-free ligand sensing assays.^{39,73} Notably, PPAR α is the only subtype that binds to a wide range of saturated fatty acids.¹⁴ All of the fatty acids identified to date bind to PPAR α with affinities in the micromolar



range. While total fatty acid levels in serum reach these concentrations,⁷⁴ it is not known whether the free concentrations of fatty acids in cells are high enough to activate the receptor. This has prompted several investigators to search for high-affinity natural ligands among the known eicosanoid metabolites of polyunsaturated fatty acids. The lipoxygenase metabolite 8(S)-HETE was identified as a higher-affinity PPAR α ligand,^{39,41,73,75} although it is not found at sufficiently high concentrations in the correct tissues to be characterized as a natural ligand. Since no single high-affinity natural ligand has been identified, we and others have proposed that one physiological role of PPAR α may be to sense the total flux of fatty acids in metabolically active tissues.^{14,26,39,41,73}



PPAR α and Dyslipidemia. The discovery that the fibrates activate PPAR α has triggered extensive research into the role of this receptor in mediating the lipid-lowering activity of these drugs. In humans, fibrates are effective at lowering serum triglycerides and raising HDL cholesterol (HDLc), primarily through increased clearance and decreased synthesis of triglyceride-rich VLDL.⁷⁶ Fibrates lower serum levels of apoC-III, a known inhibitor of VLDL clearance,⁷⁷ in both

humans⁷⁸ and rodents.⁷⁹ A PPAR response element has been identified in the proximal promoter of the *apoC-III* gene through which PPAR α can inhibit its expression.⁸⁰ Response elements have also been identified in proximal promoters of other genes involved in lipid metabolism, such as *AOX*,⁵⁰ cytochrome P450 4A,⁸¹ liver-fatty acid-binding protein (*L-FABP*),⁸² and lipoprotein lipase (*LPL*).⁸³ In rodents apoA-I expression is down-regulated by fibrate treatment, leading to a reduction in HDLc. Since rodents carry most of their cholesterol in the HDL fraction, this is one pathway by which fibrates lower cholesterol levels in rodents. In humans, however, the reverse is true: fibrate treatment increases apoA-I expression leading to a rise in HDLc levels. This difference in gene regulation may be due to variations in the response element sequences in the rodent and human *apoA-I* genes.⁸⁴⁻⁸⁶ The availability of a PPAR α null mouse has aided the study of the role of the receptor in lipid homeostasis.⁸⁷ In contrast to wild-type mice, PPAR $\alpha^{-/-}$ mice do not show decreases in serum lipid levels as a consequence of treatment with fibrates. Moreover, the null mice do not exhibit hepatomegaly, peroxisome proliferation, or induction of enzymes responsible for fatty acid oxidation when challenged with fibrates.⁸⁸

The LDL cholesterol (LDLc)-lowering activity of the current fibrate drugs is weak compared to the statin class of drugs that inhibit HMG-CoA reductase.⁸⁹ At present, LDLc lowering is the primary endpoint by which lipid-lowering therapies are compared. Data are available showing a correlation of cardiac events with increasing LDLc levels, and cardiovascular outcome studies with statins have shown improvements in patient mortality and morbidity.⁹⁰ However, recent evidence has suggested that hypertriglyceridemia and low HDLc levels should also be considered when assessing a patient's cardiovascular risk factors.⁹¹ Fibrates have been shown to slow the progression of atherosclerosis and reduce the number of coronary events in secondary prevention studies⁹²⁻⁹⁴ and in patients with normal levels of LDLc.⁹⁵ Potent subtype-selective PPAR α agonists, such as GW 9578, are more effective than the current fibrate drugs at lowering apoC-III levels in rodents.⁷¹ Thus, drugs with potent activity on human PPAR α may be useful adjuncts to current therapies for treatment of dyslipidemias in patients at risk of cardiovascular disease.

PPAR α and Atherosclerosis. Factors other than dyslipidemia contribute to the development of coronary heart disease. Evidence is emerging that PPAR α agonists may have direct effects in the arterial wall, which could contribute to the beneficial effects of these drugs in atherosclerosis prevention studies.⁹²⁻⁹⁵ Atherosclerotic lesion formation requires recruitment of monocytes into the arterial wall through expression of adhesion molecules by activated endothelial cells.⁹⁶ Expression of the adhesion molecule VCAM-1 was down-regulated by PPAR α agonists in human vascular endothelial cells.⁹⁷ This process was mediated in part by inhibition of NF- κ B, a ubiquitous transcription factor that transduces the effects of many proatherogenic and inflammatory stimuli.⁹⁸ PPAR α has also been shown to inhibit the actions of NF- κ B in aged mice. Wy-14643 corrected the abnormal expression of genes under the control of

NF- κ B in these mice⁹⁹ but was ineffective in aged $PPAR\alpha^{-/-}$ mice.¹⁰⁰ Inflammatory processes have been implicated in disruption of the atherosclerotic plaque that leads to thrombolytic events.¹⁰¹ $PPAR\alpha$ agonists were shown to inhibit the IL-1-stimulated release of IL-6 and inflammatory prostaglandins in vascular smooth muscle cells.¹⁰² Patients with coronary artery disease also responded favorably to fenofibrate treatment, showing reduced plasma levels of IL-6, fibrinogen, and C-reactive protein,¹⁰² possibly through negative regulation of NF- κ B and AP-1 by $PPAR\alpha$.¹⁰³ Additional evidence that $PPAR\alpha$ can affect inflammatory processes was generated using the classical mouse ear-swelling test. Arachidonic acid-induced ear swelling lasted longer in $PPAR\alpha^{-/-}$ mice compared to wild-type mice,¹⁰⁴ and it was hypothesized that $PPAR\alpha$ may regulate genes involved in the catabolism of inflammatory eicosanoids. In support of this proposal the ear-swelling response to phorbol myristate acetate, which is not mediated by eicosanoids, was not affected by $PPAR\alpha$ genotype.¹⁰⁴

PPAR α and Obesity/Diabetes. Obesity is a major risk factor for the development of diabetes, and fibrate treatment has been reported to reduce weight gain in rodents without effects on food intake.¹⁰⁵⁻¹⁰⁸ Notably, $PPAR\alpha^{-/-}$ mice show increased accumulation of body fat as they age.^{109,110} These observations suggest that $PPAR\alpha$ may affect body weight through regulation of fatty acid catabolism or energy expenditure.¹¹¹ Uncoupling proteins (UCP) 1-3 are mitochondrial membrane transporters that uncouple substrate oxidation from ATP synthesis, allowing conversion of fuel into heat.¹¹² Examination of the changes in UCP mRNA expression following bezafibrate treatment of rodents revealed increased levels of UCP1 in white adipose tissue (WAT) and increased levels of UCP3 in WAT and skeletal muscle.¹¹³ In rat neonates Wy-14643 has been shown to induce the expression of *UCP3*,¹¹⁴ a gene whose message levels are reduced in type 2 diabetics.¹¹⁵ Three putative PPAR response elements have been identified in the *UCP3* promoter sequence.¹¹⁶ Wy-14643 has been shown to transactivate the *UCP1* promoter in fetal rat brown adipocytes, although this did not result in increased UCP1 mRNA levels.¹¹⁷ Under these same conditions, the PPAR γ agonist rosiglitazone activated the *UCP1* promoter and increased UCP1 mRNA levels, but a combination of the $PPAR\alpha$ and PPAR γ agonists did not lead to increased UCP1 expression.¹¹⁷ Interestingly, clofibrate and bezafibrate have been shown to improve glucose tolerance in type 2 diabetic patients.¹¹⁸⁻¹²⁰ However, the clinically used fibrates are only moderately selective for $PPAR\alpha$ over PPAR γ ; thus it is not clear whether activation of the latter subtype is responsible for the observed effects. Confirmation of the therapeutic potential of $PPAR\alpha$ agonists in obesity or diabetes will require clinical testing of drugs with greater subtype selectivity than the currently available fibrates.

PPAR γ

PPAR γ is the most extensively studied of the three PPAR subtypes to date. The receptor has been cloned from a number of species, including salmon,¹²¹ mice,^{15,122} hamsters,¹²³ frogs,⁵⁰ pigs,¹²⁴ rhesus monkeys,¹²⁵ and humans.¹²⁶⁻¹²⁸ The human PPAR γ protein is homolo-

gous to the murine PPAR γ protein, with 95% identity at the amino acid level. In fact, the PPAR γ protein shows a remarkable conservation across all the species from which it has been cloned, in contrast to that found thus far for $PPAR\alpha$ and $PPAR\delta$. This high level of conservation may reflect the pivotal role that PPAR γ plays as a regulator of glucose and lipid homeostasis, essential functions across all species. The human *PPAR γ* gene has nine exons that extend over more than 100 kb of genomic DNA and has been mapped to human chromosome 3p25 by somatic cell hybridization and linkage analysis.^{126,129}

Three mRNA isoforms of PPAR γ have been detected in humans (termed PPAR γ 1, PPAR γ 2, and PPAR γ 3) which arise as products of different promoter usage.¹³⁰⁻¹³² PPAR γ 1 and PPAR γ 3 mRNAs code for the same protein, while PPAR γ 2 codes for a different protein containing 28 additional amino acids at the N-terminus. Analysis of the tissue distribution of the three human PPAR γ isoforms revealed some differences. PPAR γ 1 had the broadest tissue expression, while the PPAR γ 2 and PPAR γ 3 isoforms had a more restricted distribution. Thus, PPAR γ 2 was expressed predominantly in adipose tissue,¹³⁰ and PPAR γ 3 was found only in adipose tissue, macrophages, and colon epithelium.^{132,133} In addition to the aforementioned tissues, PPAR γ 1 mRNA was found in the heart, large and small intestines, colon, kidney, pancreas, spleen, and skeletal muscle. Cell culture experiments have demonstrated that PPAR γ 2 mRNA was induced during the early stages of adipocyte differentiation,¹³⁴⁻¹³⁶ but PPAR γ 1 mRNA remained unchanged.¹³⁷ However, the functional significance of the multiple isoforms of PPAR γ is currently unclear.^{131,136,138}

PPAR γ is a critical transcription factor in the regulation of adipocyte differentiation. Forced expression of PPAR γ in fibroblasts in the presence of weak PPAR γ activators resulted in differentiation of the cells to adipocytes.¹³⁵ Adipogenesis appears to require coordinated expression of the PPAR γ and two other groups of transcription factors, C/EBP and ADD-1/SREBP-1. The detailed aspects of the interplay between PPAR γ and these other transcription factors in adipocyte differentiation have been reviewed recently.^{139,140} Adipocyte differentiation is accompanied by the induction of several genes involved in lipid homeostasis such as *aP2*,¹³⁶ *PEPCK*,¹⁴¹ acyl-CoA synthetase (ACS),¹⁴² and *LPL*.⁸³ All of these genes have been shown to contain PPAR response elements in their regulatory regions. PPAR γ has also been shown to up-regulate the expression of the fatty acid transporters FATP-1 and CD36 in adipocytes.^{143,144} These data demonstrate that PPAR γ plays a pivotal role in the adipogenic signaling cascade and also suggest that the receptor can influence the production and cellular uptake of its own activators.

PPAR γ has been shown to modulate a number of other genes involved in energy storage and utilization. Activation of PPAR γ represses the expression of the *ob* gene, which codes for leptin.^{145,146} PPAR γ also appears to oppose the effects of another important adipocyte signaling factor, TNF α . Treatment of obese animals with PPAR γ agonists reduces adipose tissue expression of TNF α .¹⁴⁷ PPAR γ has been shown to up-regulate expression of the mitochondrial uncoupling proteins

UCP1, UCP2, and UCP3 in cells^{148–151} and animals.^{152,153} Recently, it was shown that activation of PPAR γ in cultured adipocytes induced expression of c-Cbl associated protein, a potential signaling protein in insulin action.¹⁵⁴ The growing list of genes that are modulated by PPAR γ continues to reinforce the notion that PPAR γ is a key regulator of adipocyte function and systemic lipid homeostasis.

Synthetic Agonists. The first compounds reported as high-affinity PPAR γ agonists were a class of antidiabetic agents known as thiazolidinediones (TZDs) or "glitazones".²⁷ The TZDs had been developed over a period of 15 years through empirical compound screening in rodent models of insulin resistance.¹⁵⁵ The molecular mechanism of action of the TZDs remained unknown until several reports in the mid-1990s suggested a possible connection between these agents and the PPARs. TZDs had been shown to induce gene expression in adipocytes and to enhance adipocyte differentiation in cell culture.^{156,157} The TZD pioglitazone was also shown to increase expression of the *aP2* gene,¹⁵⁸ through a region of its promoter that had been independently identified by the Spiegelman group as a PPAR γ binding site.¹³⁶ Through the use of binding and transactivation assays, the TZD rosiglitazone (BRL 49653) was identified as a high-affinity subtype-selective agonist for PPAR γ .^{27,159} In addition, the rank order of PPAR γ potency of a number of TZDs has been shown to closely match their glucose-lowering activity in rodents.^{42,160} These latter two pieces of data provide compelling evidence that PPAR γ is the major receptor mediating the antidiabetic activity of the TZDs.

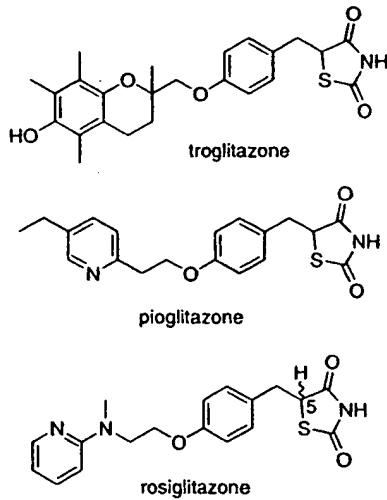
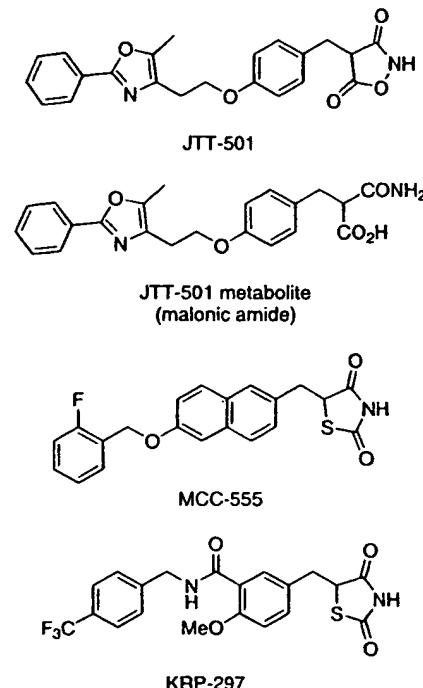


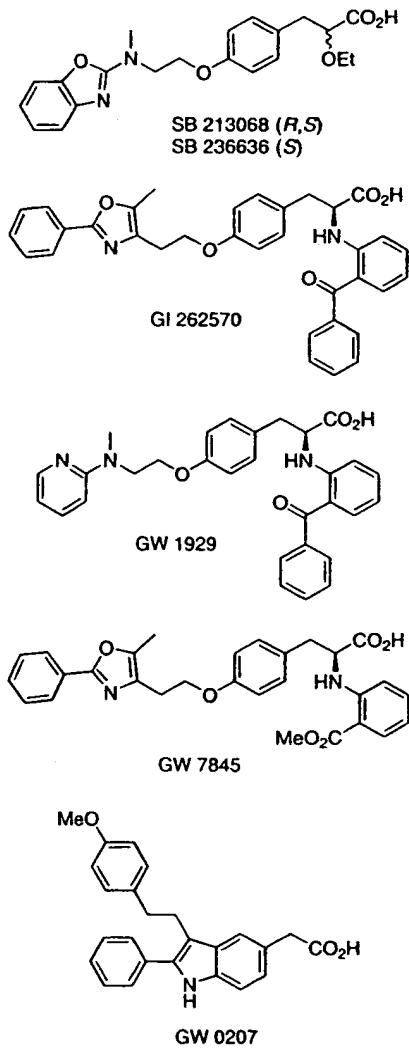
Table 1 lists the potency of those TZDs whose PPAR γ activity has been reported. TZDs, in general, are selective for PPAR γ over the PPAR α and PPAR δ subtypes,^{27,160} although a TZD, KRP-297, with agonist activity at PPAR α and PPAR γ was recently disclosed.^{161–163} The structurally related isoxazolidinedione JTT-501 has also been reported to activate PPAR α at concentrations approximately 10-fold higher than those required for activation of PPAR γ .¹⁶⁴ Its activity is likely to be mediated through a malonic amide metabolite that is generated by hydrolysis of the heterocyclic ring.¹⁶⁵ The TZD MCC-555 was reported to be a low-affinity but high-efficacy PPAR γ agonist that showed potent an-

tidiaabetic activity in rats.^{166,167} Interestingly, MCC-555 appeared to function as a full or partial PPAR γ agonist depending on the cell type and the response element used in the transactivation assay.¹⁶⁸ A series of antidiabetic Δ^5 -unsaturated-TZDs have also been reported, which were claimed to show little or no activity at PPAR γ .^{169,170} These observations raise the possibility that some TZDs mediate their antidiabetic activity through mechanisms other than PPAR γ , although activation of PPAR γ by metabolites of these drugs cannot be ruled out. An alternative explanation is that these compounds are able to produce tissue or promoter-specific modulation of PPAR γ target genes, which was not detected by the standard PPAR γ transactivation assays. Should this latter hypothesis prove valid, it will add a new dimension to the development of PPAR γ ligands, similar to that stimulated by the selective estrogen receptor modulators in the osteoporosis field.¹⁷¹



The TZDs contain a stereogenic center at C-5 of the heterocyclic headgroup but have been developed as racemates since they undergo racemization under physiological conditions.¹⁷² Using a PPAR γ binding assay it has been shown that only the (S)-enantiomers of the TZDs bind to the receptor with high affinity.¹⁷³ This surprising result suggests that only 50% of the drug substance in the currently approved TZDs binds to the target receptor, while 50% of the drug substance is inactive. To overcome this problem several groups have identified acyclic headgroups that are less prone to racemization. SB 213068 and its (S)-isomer, SB 236636, are representatives of a series of α -alkoxy- β -phenylpropanoic acids^{174,175} that show agonist activity at PPAR γ and PPAR α (Table 1). Within this series the (S)-enantiomers were shown to have higher binding affinity for PPAR γ and were more potent than their (R)-enantiomers in adipocyte differentiation assays.^{43,176} Recently we reported a series of tyrosine-based PPAR γ agonists exemplified by GI 262570, GW 1929, and GW

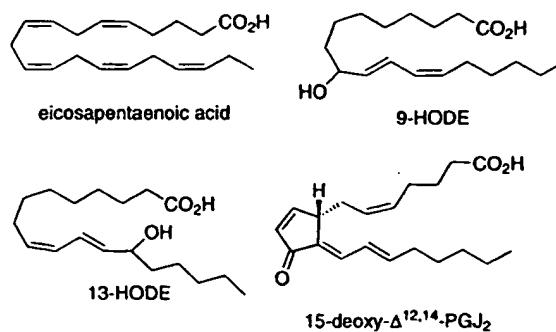
7845.¹⁷⁷⁻¹⁷⁹ These compounds were the first antidiabetic drugs to be optimized based on their activity at human PPAR γ . This series also contains some of the most potent PPAR γ agonists reported to date, with a number of analogues having sub-nanomolar activity at human PPAR γ (Table 1). In addition, these compounds showed >1000-fold selectivity for PPAR γ over the PPAR α and PPAR δ subtypes in cell-based transactivation assays. GW 1929 demonstrated antihyperglycemic activity equal to troglitazone at >100-fold lower plasma concentrations in ZDF rats,^{177,180} which parallels their differences in PPAR γ binding and activation. Within this series the (S)-enantiomers, synthesized from naturally occurring L-tyrosine, were shown to have greater binding affinity and functional activity at PPAR γ than the corresponding (R)-enantiomers.¹⁷⁷



Other structurally diverse PPAR γ agonists have been described in the literature. The 2,3-disubstituted indole-5-acetic acid derivative GW 0207 was a potent and selective PPAR γ agonist.¹⁸¹ Berger⁴⁵ has reported a series of phenylacetic acid derivatives, such as L-796449, which showed potent activity at PPAR γ . Notably, L-796449 activated human PPAR α and PPAR δ at similar concentrations (Table 1), the first reported example of a potent agonist across all three receptor

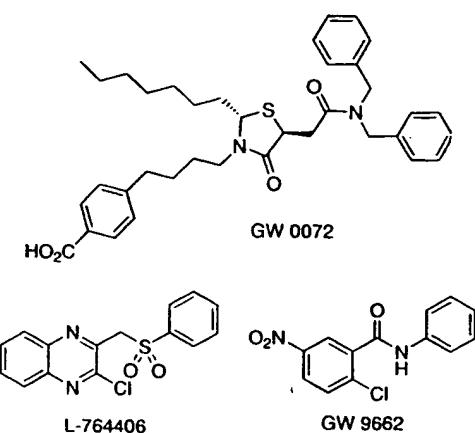
subtypes. Several inhibitors of eicosanoid biosynthesis or receptor signaling pathways have also been reported to be weak agonists at PPAR γ . The LTD₄ receptor antagonist LY 171883 activated PPAR γ in transactivation assays at micromolar concentrations.^{15,159} The cyclooxygenase inhibitor indomethacin has been shown to bind and activate PPAR γ at high micromolar concentrations.¹⁸² Indomethacin was shown to promote adipocyte differentiation at concentrations similar to that required for activation of PPAR γ .^{183,184} Several other NSAIDs, including ibuprofen, fenoprofen, and flufenamic acid, were also shown to be weak PPAR γ agonists.¹⁸² It is important to note that interaction of these compounds with PPAR γ occurs at higher concentrations than is required for inhibition of cyclooxygenase or LTD₄ receptor antagonism.

Natural Ligands. The search for the natural PPAR γ ligands has led to the discovery of a number of fatty acids and eicosanoid derivatives that bind and activate the receptor at micromolar concentrations. Unlike the PPAR α subtype, PPAR γ has a clear preference for polyunsaturated acids.¹⁴ The essential fatty acids linoleic acid, linolenic acid, arachidonic acid, and eicosapentaenoic acid (EPA) have been shown to bind PPAR γ at micromolar concentrations.^{14,41} These values are within the range of concentrations of free fatty acids found in human serum;⁷⁴ however, as discussed earlier, it is unclear if these concentrations of fatty acids exist within cells. In fact, fatty acids are not particularly efficacious activators of PPAR γ , and it has been demonstrated that intracellular conversion of fatty acids to eicosanoids, through enhanced expression of 15-lipoxygenase, resulted in increased PPAR γ -mediated transactivation.¹⁸⁵ The 15-lipoxygenase metabolites of linoleic acid, 9-HODE and 13-HODE, have been shown to function as micromolar PPAR γ agonists.¹⁸⁶ Therefore, metabolic conversion of polyunsaturated fatty acids within cells could provide an additional level of hormonal regulation of PPAR γ . The J-series of prostaglandins derived from PGD₂ have also been identified as PPAR γ ligands.⁷⁵ The terminal metabolite 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) activated PPAR γ at low micromolar concentrations and also induced adipocyte differentiation.^{159,187} This prostaglandin has become the most widely utilized naturally occurring PPAR γ ligand. However, it is important to note that 15d-PGJ₂ mediates some of its effects in cells through PPAR γ -independent signaling pathways,¹⁸⁸⁻¹⁹¹ and caution must be exercised in attributing the biological effects elicited by 15d-PGJ₂ solely to activation of PPAR γ .



Partial Agonists and Antagonists. A novel PPAR γ ligand GW 0072, which was identified from a diverse

combinatorial library, profiled as a partial agonist in transactivation assays and was an inhibitor of adipocyte differentiation in cell culture.¹⁹² This compound had only 15–20% of the efficacy of rosiglitazone and was able to antagonize rosiglitazone in transactivation assays to the level of its own partial agonist activity with an $IC_{50} = 150$ nM. The low efficacy of GW 0072 was paralleled by its reduced ability to recruit coactivator proteins CBP and SRC1 to the receptor, which appears to be the result of a novel binding mode of the ligand within the PPAR γ LBD (see below).¹⁹² Although not a pure antagonist, GW 0072 may provide a valuable chemical tool for dissecting the pharmacology of PPAR γ . Scientists at Merck have described a partial agonist, L-764406, which is an irreversible PPAR γ ligand.⁴⁷ L-764406 binds covalently to Cys²⁸⁵ on helix 3 of the PPAR γ LBD (Cys³¹³ in PPAR γ 2). L-764406 displayed approximately 25% of the maximal activity obtained with TZDs, both in transactivation assays and in the induction of aP2 expression in 3T3-L1 preadipocyte cells. Antagonist activity of this compound was not reported. However, we have shown that an irreversible PPAR ligand, GW 9662, was able to antagonize the activation of PPAR γ in macrophages.¹⁸⁵



Protein Structure. The structure of the PPAR γ LBD has been determined by X-ray crystallography. Structures have been solved in the absence of ligand (apo-PPAR γ)^{12,13} (Figure 2A) and bound to the TZD rosiglitazone together with an 88-amino acid fragment of the coactivator SRC1 (Figure 3).¹² The PPAR γ LBD is a bundle of 13 α -helices and a small four-stranded β -sheet (Figure 2A), with an overall fold similar to other nuclear receptor structures from helix 3 to the C-terminus.¹⁹³ The crystal structure of the apo-PPAR γ LBD revealed a large (~ 1300 \AA^3) Y-shaped ligand-binding site located within the bottom half of the LBD, extending from the C-terminal α -helix (known as AF-2) to the β -sheet between helices 3 and 6. The presence of an additional helix 2' (Figure 2A), which is not seen in the steroid, retinoid, or thyroid receptor LBDs, may be responsible for the large volume of the PPAR γ ligand-binding site.^{12,13} Rosiglitazone binds in a U-shaped conformation, while occupying only 40% of the ligand-binding site. The TZD headgroup makes several specific hydrogen-bonding interactions with His⁴⁴⁹, Tyr⁴⁷³, His³²³, Ser²⁸⁹, and Gln²⁸⁶ (Figure 4A). Tyr⁴⁷³ lies on the C-terminal AF-2 helix, which is critical for transcriptional

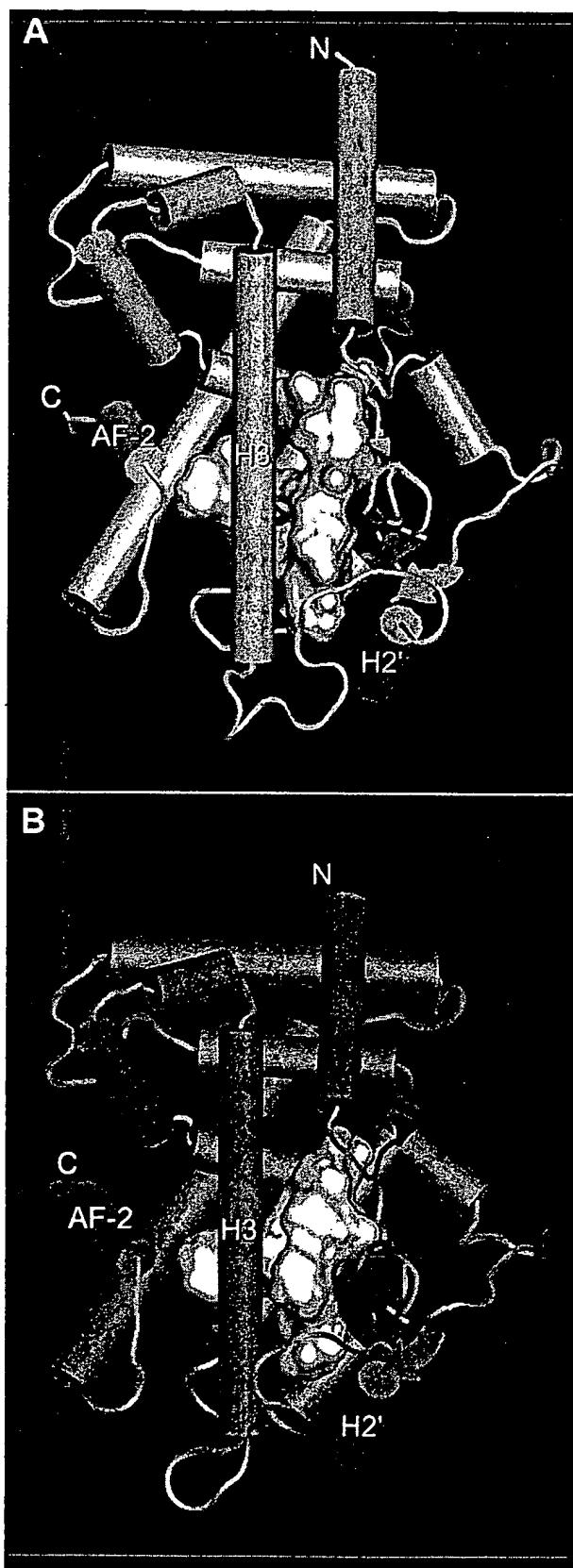


Figure 2. Crystal structures of the apo-PPAR LBDs. The α -helices are represented by tubes. Helix 2', helix 3, and the AF-2 helix are designated as H2', H3, and AF-2, respectively. The solvent-accessible ligand-binding site is indicated by the white shaded surface: A. PPAR γ LBD; B. PPAR δ LBD.

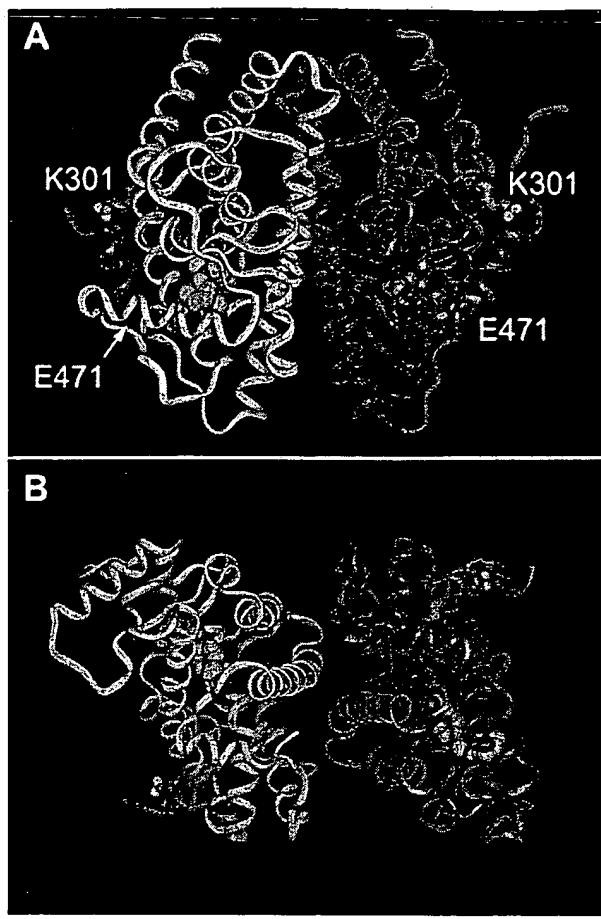


Figure 3. Crystal structure of the ternary complex of the PPAR γ LBD with rosiglitazone and SRC1. Each PPAR γ LBD of the homodimer is shown as a yellow worm. The side chains of the charge clamp residues Lys³⁰¹ and Glu⁴⁷¹ are colored orange. The SRC1 fragment containing the LxxLL motifs is shown in purple, but the linking peptide which showed poor electron density has been omitted. The TZD ligand is color coded by atom: A. side view of the complex, Lys³⁰¹ and Glu⁴⁷¹ are indicated; B. bottom view of the complex.

activation and provides the first example of a nuclear receptor ligand that interacts directly with a residue in this helix. Interestingly, in both the apo and ligand-bound structures of PPAR γ , the AF-2 helix adopted a conformation similar to other ligand-bound nuclear receptors.¹⁹³ Although these structures do not provide clear evidence of a ligand-induced conformational change of the AF-2 helix, it is likely that interaction of the ligand with this helix locks the receptor in an activated state.

The ternary complex of PPAR γ with rosiglitazone and SRC1 revealed the receptor crystallized as a homodimer bridged by a single molecule of the coactivator (Figure 3).¹² The homodimer is likely to use the same dimer interface as the physiologically relevant PPAR γ /RXR heterodimer.²³ Each receptor in the dimer interacts with a short α -helix from the SRC1 fragment through a 'charge clamp' formed by Glu⁴⁷¹ on AF-2 and Lys³⁰¹ on helix 3 (Figure 3A). Each α -helix in the SRC1 fragment contains the peptide sequence LxxLL, where L = Leu and x = any amino acid. The charge clamp places the LxxLL motif into the optimal orientation to bury its Leu residues into a hydrophobic cleft on the surface of the

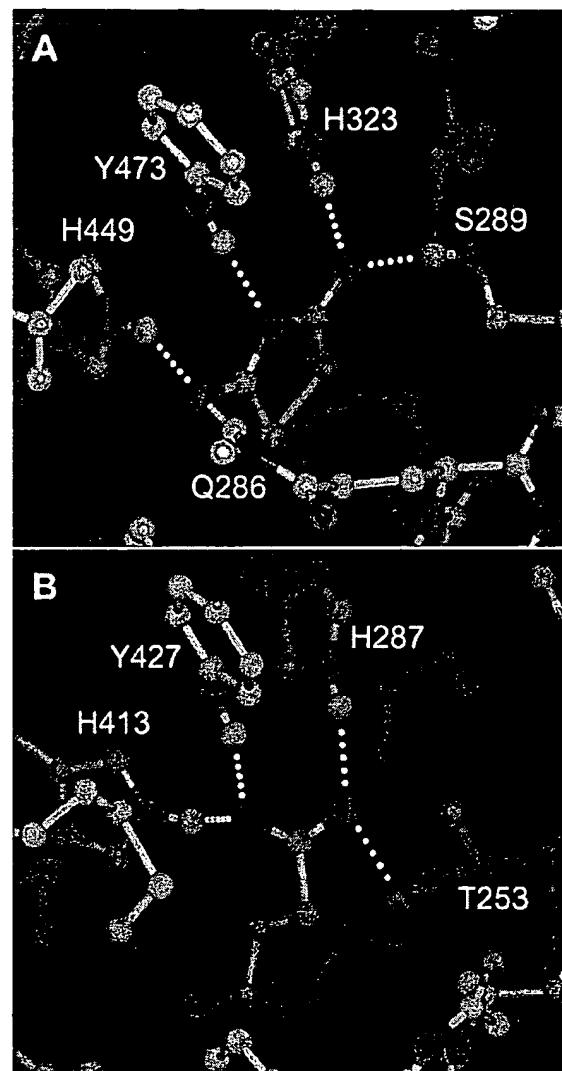


Figure 4. Conserved hydrogen-bonding interactions of PPAR agonists with the LBD. The key amino acids are indicated with their hydrogen bonds to the ligand drawn as white broken lines: A. TZD headgroup of rosiglitazone bound to PPAR γ ; B. carboxylic acid headgroup of EPA bound to PPAR δ .

receptor (Figure 3B). Both LxxLL motifs make identical contacts with the receptor dimer, which may allow the multiple LxxLL motifs found in most coactivator proteins to mediate cooperative binding to nuclear receptor dimers. These structures revealed that binding of the PPAR ligand directly to the AF-2 helix stabilized the formation of the charge clamp on the surface of the receptor, which in turn is critical for recruitment of coactivator proteins to the receptor complex through their LxxLL motifs. Further support for this mechanism comes from the X-ray crystal structure of PPAR γ bound to the partial agonist GW 0072.¹⁹² In this structure (Figure 5), GW 0072 has a binding mode in which the ligand does not contact the AF-2 helix.¹⁹² Although the backbone of the AF-2 helix was observed in the agonist-bound conformation, Tyr⁴⁷³, His³²³, and His⁴⁴⁹ adopted side-chain conformations similar to those in the apo-PPAR γ LBD crystal structure.¹⁹² This suggests that in the GW 0072-bound receptor the charge clamp would not be stabilized through direct interactions with the ligand, which may account for the weak recruitment of



Figure 5. Partial agonist GW 0072 has a different binding mode to PPAR γ compared to rosiglitazone. PPAR γ LBD from the cocrystal with GW 0072 is shown as a yellow worm. GW 0072 is shown with its carbon atoms colored blue. Rosiglitazone was superimposed on the structure by alignment of the corresponding PPAR γ LBD protein (not displayed). Rosiglitazone is shown with its carbon atoms colored green.

coactivator proteins.¹⁹² Thus, it appears that the large binding pocket of PPAR γ is not only capable of binding a number of structurally diverse ligands (Figure 5) but can also be exploited to identify ligands with altered binding characteristics and modified receptor pharmacology.

PPAR γ and Diabetes. The most extensively studied therapeutic utility for PPAR γ agonists has been in the treatment of type 2 diabetes.¹⁹⁴ TZD PPAR γ agonists have been shown to enhance the sensitivity of target tissues to insulin and to reduce plasma glucose, lipid, and insulin levels in animal models of type 2 diabetes as well as in humans.^{195–199} Troglitazone (Rezulin), pioglitazone (ACTOS), and rosiglitazone (Avandia) have been approved by the FDA and are currently marketed agents for the treatment of type 2 diabetes. These drugs represent an important advance in the treatment of this disease when used as monotherapy or in combination with existing therapies. Troglitazone has produced significant reductions in plasma glucose, HbA_{1c}, insulin, and C-peptide levels, either alone or in combination with sulfonylureas or metformin in type 2 diabetics.^{200–202} In addition, troglitazone therapy has been shown to have beneficial effects on serum lipids,²⁰³ blood pressure, and cardiac output.²⁰⁴ However, a significant number of diabetics fail to respond to troglitazone therapy, and concerns have been expressed about the potential for weight gain^{205,206} or increased adipogenesis in bone marrow²⁰⁷ that is seen with TZDs in rodents.

A much more serious issue with troglitazone is hepatotoxicity. Troglitazone has been linked to a number of cases of liver failure²⁰⁸ that has resulted in its

withdrawal for use as monotherapy in newly diagnosed type 2 diabetics in the United States and withdrawal of the drug from the market in the United Kingdom. It is presently unclear whether the observed hepatotoxicity is mediated by PPAR γ or by some other mechanism unique to troglitazone. Neither rosiglitazone nor pioglitazone has displayed the increased incidence of hepatic adverse events seen with troglitazone, suggesting that hepatotoxicity may not be a class effect of PPAR γ agonists. Troglitazone has also been shown to activate the human pregnane X receptor (PXR) [NR1I2]¹¹ at the concentrations needed to activate PPAR γ .²⁰⁹ PXR is a recently isolated orphan member of the nuclear receptor gene family that was shown to be a key transcriptional regulator of hepatic cytochrome P450 3A4 (CYP3A4) gene expression.^{210–213} Since CYP3A4 is responsible for the oxidative metabolism of around 60% of all clinically used drugs, activation of PXR may be the molecular mechanism underlying the observed drug–drug interactions of troglitazone.^{214,215} Troglitazone is also a substrate for CYP3A4, leading to formation of a potentially reactive quinone metabolite.^{216,217} Thus, the cross-reactivity of troglitazone on PXR may lead to increased hepatic levels of the quinone metabolite in some patients. In addition, other known activators of PXR cause hepatomegaly in rats, suggesting that PXR may regulate additional genes involved in liver metabolism.²¹⁰ PXR is remarkably divergent across species, and troglitazone does not activate the mouse or rat receptor.²⁰⁹ This may explain why hepatotoxicity was not seen in the rodent safety studies of the drug. While it remains to be proven that activation of PXR by troglitazone is responsible for the hepatotoxicity in humans, screening of new PPAR γ agonists for selectivity against PXR should lead to the development of diabetes drugs with reduced propensity for P450 enzyme induction and drug–drug interactions.²⁰⁹

RXR agonists are also known to activate the PPAR γ /RXR heterodimer and can show additive activity with PPAR agonists in cell-based transactivation assays.^{23,218} Interestingly, targretin (LGD1069) and LG100268, selective RXR agonists that had been developed primarily as anticancer drugs, have shown glucose- and lipid-lowering activity in rodents.^{218,219} These effects are likely to be mediated through activation of the PPAR γ /RXR and PPAR α /RXR heterodimers, although the activation of other RXR heterodimers cannot be ruled out. Notably, reports from human clinical trials indicate that these drugs may raise serum triglycerides²²⁰ or lead to alterations in thyroid metabolism.^{221,222} Thus, while it is still unclear that RXR agonists will be used as monotherapy for diabetes, they may be useful in combination therapy to increase the glucose- or lipid-lowering activity of various PPAR agonists.

The discovery that PPAR γ is the molecular target of the antidiabetic TZDs has raised two apparent paradoxes. First, how can a receptor expressed mainly in adipose tissue improve insulin sensitivity and glucose utilization in skeletal muscle, the major insulin-sensing tissue? Several explanations have been proposed. Troglitazone has been shown to ameliorate insulin resistance and hyperglycemia in mice engineered to lack adipose tissue, suggesting that PPAR γ action can occur by adipocyte-independent pathways.²²³ Although PPAR γ

levels are 10–100-fold higher in adipose than in muscle or liver, the receptor is expressed in these latter tissues.^{131,224–226} Thus, stimulation of PPAR γ in these non-adipose tissues could contribute to altered gene expression and a reduction in insulin resistance and improved glucose disposal. PPAR γ may also exert direct effects on genes involved in glucose homeostasis, although surprisingly little is known about this topic. One potential target gene for PPAR γ that is central to improving muscle glucose disposal is the insulin-dependent glucose transporter GLUT4. TZD activation of PPAR γ has been shown to increase the expression of this gene in adipocytes,^{227,228} but direct regulation of its expression in muscle has not been reported. UCP2, a mitochondrial uncoupling protein related to UCP1, has been shown to be expressed in a number of tissues including skeletal muscle and adipose tissue and appears to function as an important modulator of energy usage.²²⁹ Expression of UCP2 was up-regulated by TZD activation of PPAR γ in cell lines derived from skeletal muscle, WAT, and brown adipose tissue (BAT).^{148,150} Finally, PPAR γ agonists may regulate the storage or release adipocyte-derived signaling factors that affect insulin sensitivity in muscle. Fatty acids are key mediators of this process. It is well-established that increased fatty acid concentrations decrease glucose metabolism in muscle.²³⁰ While PPAR γ agonists induce LPL, FATP-1, and ACS in adipose tissue, their expression is apparently unchanged in muscle.²³¹ This may allow for an increase in fatty acid clearance into adipose tissue with concomitant decrease in uptake of fatty acids into muscle, potentially improving insulin sensitivity.²³⁰ In support of this hypothesis TZD treatment is reported to reduce the triglyceride content of muscle in diabetic rats.²³² The mechanism of these effects may be related to the observation that PPAR γ regulates expression of the fatty acid transporter CD36,²³³ which was implicated in the control of insulin sensitivity in rats.²³⁴ PPAR γ -induced differentiation in WAT is also associated with a marked decrease in the levels of TNF α and leptin, two signaling molecules that are secreted by adipocytes.^{235,236} Elevated levels of TNF α can cause insulin resistance,²³⁷ and TZDs have been shown to block TNF α -induced insulin resistance in both cell culture²³⁸ and animals.²³⁹

The second apparent paradox lies in the fact that obesity is a major risk factor in the development of type 2 diabetes. How then is a receptor that promotes adipogenesis still able to enhance insulin sensitivity? Evidence suggests that PPAR γ -mediated differentiation of white adipocytes in rodents produces an increased number of small adipocytes while decreasing the number of large adipocytes.^{235,236} These smaller adipose cells are usually more sensitive to insulin and would be expected to provide greater insulin-dependent glucose uptake. The smaller adipose cells should also have lower rates of lipolysis relative to larger adipose cells. Since high levels of free fatty acids have been linked to the induction of insulin resistance,^{230,240,241} the decreased amount of circulating free fatty acid levels would be expected to have beneficial effects on insulin sensitivity. TZDs also induce the differentiation of BAT in rodents¹⁵² and induce the expression of the mitochondrial uncoupling protein gene UCP1 in human primary

adipocytes.²⁴² These effects may also contribute to increased energy consumption and decreased glucose and lipid levels.

Several polymorphisms have been identified in the human PPAR γ gene. A rare Pro¹¹⁵Gln mutation in the PPAR γ 2 N-terminal domain, resulting in a constitutively active receptor, was identified in four obese German individuals.²⁴³ This phenotype was consistent with PPAR γ playing a role in the regulation of fat storage in humans. A more common silent C/T-polymorphism in exon 6 was detected in a sample of a northern French population that was associated with higher plasma leptin levels in obese subjects but not body mass index (BMI).²⁴⁴ A relatively common Pro¹²Ala mutation in the PPAR γ 2-specific exon B has also been identified independently by two research groups.^{245,246} Deeb found this mutation was associated with lower BMI, improved insulin sensitivity, and higher plasma HDLc concentrations.²⁴⁶ The association with insulin sensitivity disappeared when corrected for BMI, indicating that the mutation primarily affects body weight. From a population of obese Finns, two women who were severely overweight were found to carry both the exon B and exon 6 polymorphisms.²⁴⁷ However, other reports have found no association of the Pro¹²Ala mutation with BMI²⁴⁸ and insulin sensitivity.²⁴⁹ Subsequent reports^{250–253} have failed to resolve this apparent conflict, underscoring the influence of environmental factors and the difficulty in determination of these phenotypes in humans.

In contrast to the human genetic studies, recent reports^{254–256} of the phenotype of the PPAR γ null mouse have shed additional light on the role of PPAR γ in adipocyte function and insulin resistance. The PPAR γ null mutation was embryonic lethal around day 10 of gestation due to a defect in placental development.^{254,255} As expected, PPAR γ ^{-/-} embryonic stem cells and fibroblasts failed to undergo differentiation to a mature adipocyte phenotype in cell culture.^{255,256} Using a placental rescue technique Barak showed that PPAR γ was not essential for further development of the mice.²⁵⁴ A single mouse that was brought to full-term lacked adipose tissue and showed extreme metabolic defects, similar to the human condition of lipodystrophy.²⁵⁷ These results confirmed the essential role of PPAR γ in the formation of mature adipocytes and its important role in lipid and glucose homeostasis mediated through adipose tissue. Notably, the heterozygous PPAR γ ^{+/-} mice develop normally, have a normal number and size of adipocytes, and show no metabolic defects. In an elegant experiment, Kubota reported that upon challenge with a high-fat diet, the PPAR γ ^{+/-} mice were partially protected from weight gain and the development of insulin resistance when compared to their wild-type litter mates.²⁵⁵ This surprising result was explained by the observation that the adipocytes of the PPAR γ ^{+/-} mice were less hypertrophic, and the circulating leptin levels were 2-fold higher than in the wild-type mice. UCP expression was also 20% higher in the BAT of the PPAR γ ^{+/-} mice. The differences were consistent with the decreased food intake and higher basal metabolic rate that was observed in the PPAR γ ^{+/-} mice. These results raise the intriguing possibility of a dichotomy in PPAR γ function with respect to insulin

sensitization.²⁵⁵ On the one hand, PPAR γ activation may promote adipocyte differentiation and the formation of small adipocytes leading to increased insulin sensitivity. On the other hand, PPAR γ activation may cause hyperplasia of preexisting adipocytes and suppression of leptin production,²⁵⁸ with a consequent decrease in insulin sensitivity. Thus, it is possible that what was once a thrifty gene, which evolved to aid the storage and release of fat during cycles of feast and famine, may now predispose modern humans to obesity and type 2 diabetes in our time of relative caloric excess.²⁵⁹

PPAR γ and Dyslipidemia. It is well-established that PPAR γ agonists decrease plasma levels of triglycerides, cholesterol, and nonesterified fatty acids in various animal models of dyslipidemia.¹⁵⁵ These lipid changes are also seen in humans, although the effects are less consistent. There appear to be some minor differences among the three marketed PPAR γ agonists, although not enough clinical data are available yet on rosiglitazone and pioglitazone to afford a clear comparison. Troglitazone has shown a consistent decrease in plasma triglycerides (13–32%) and free fatty acids (up to 22%), while effects on cholesterol metabolism are variable, with some clinical trials reporting an increase in both HDLc (up to 16%) and LDLc (up to 13%) while others report no change in cholesterol levels.^{202,260} In clinical trials, rosiglitazone has shown variable results on triglyceride lowering but appears to lower free fatty acids while raising both HDLc and LDLc.²⁶¹ Pioglitazone also lowers triglycerides and raises HDLc; however, LDLc does not appear to increase upon pioglitazone treatment, with levels being no different than placebo.²⁶² The origin of the observed differences in lipid effects among these TZDs remains unclear. On the basis of the current data, PPAR γ agonists would appear to have some utility in lowering triglycerides and free fatty acids. However, no clinical trials with selective PPAR γ agonists have been conducted on patient populations that are hyperlipidemic but not diabetic, so the pharmacological profile of these agents in a strictly dyslipidemic setting is unknown.

PPAR γ and Hypertension. Hypertension is increasingly recognized as a complex metabolic and cardiovascular disorder. The pathogenesis of the disease involves improper regulation of blood pressure, insulin sensitivity, vascular function, and lipid metabolism.²⁶³ TZDs have been shown to decrease blood pressure in a number of animal models, including Dahl S rats,²⁶⁴ obese Zucker rats,^{265,266} spontaneously hypertensive rats,²⁶⁷ Watanabe rabbits,²⁶⁸ and obese insulin-resistant rhesus monkeys.²⁶⁹ Pioglitazone was reported to decrease blood pressure in the one-kidney, one-clip Sprague–Dawley rat, an animal model of hypertension not associated with insulin resistance.^{270,271} This suggests that the ability of PPAR γ agonists to prevent hypertension may be independent of their ability to improve insulin resistance. Troglitazone has been shown to reduce blood pressure in nondiabetic and diabetic humans²⁰² and improve overall cardiac output and stroke volume without an increase in cardiac mass.²⁰⁴ The mechanism by which PPAR γ agonists exert their antihypertensive effects is not well-understood. TZDs have been shown to inhibit growth factor-induced vascular smooth muscle

cell proliferation in cell culture^{264,272} and in animals,^{273,274} potentially through PPAR γ -mediated inhibition of MAP kinase.^{275,276} PPAR γ agonists may also decrease blood pressure by affecting vascular contractility.²⁷⁷ TZDs have been reported to modulate the production of several key factors involved in maintenance of vascular tone, including type-C natriuretic peptide,²⁷⁸ endothelin,^{278,279} and PAI-1.^{280,281} Finally, TZDs have been shown to modulate currents in a number of ion channels. Troglitazone, pioglitazone, and rosiglitazone all suppress the voltage-gated (L-type) Ca²⁺ current in rat aortic myocytes.^{282,283} However, the rank order of potency for Ca²⁺ channel inhibition does not correlate with potency for PPAR γ activation, which suggests that the effect of these compounds on ion channels may be mediated through PPAR γ -independent mechanisms.

PPAR γ and Inflammation. The potential role of PPAR γ in regulation of inflammatory processes was suggested by studies in adipose tissue, in which a general antagonism was seen between PPAR γ and the proinflammatory cytokine TNF α . PPAR γ agonists have been shown to reduce the expression level of TNF α in the adipose tissue of obese rats and to block the inhibitory effects of TNF α on insulin signaling²³⁸ and TNF α -induced glycerol and free fatty acid release.²⁸⁴ Monocytes and macrophages have a well-established involvement in inflammatory processes, including vascular wall inflammation and atherogenesis, through production of nitric oxide (NO) and inflammatory cytokines such as TNF α , IL-1, and IL-6. Chinetti²⁸⁵ found that PPAR γ expression was induced upon differentiation of human monocytes into macrophages. Activation of PPAR γ by rosiglitazone or 15d-PGJ₂ resulted in induction of apoptosis due to interference with the NF- κ B signaling pathway. Activation of PPAR γ in macrophages has been shown to inhibit MMP-9 activity.²⁸⁶ Treatment of activated macrophages with PPAR γ agonists resulted in a change in cell morphology and suppression of NO production.²⁸⁷ PPAR γ agonists, including certain NSAIDs, block the production of inflammatory cytokines in human monocytes treated with phorbol myristate acetate.²⁸⁸ However, it is important to point out that in both of these latter studies, the antiinflammatory activity seen with rosiglitazone occurred at concentrations considerably higher than the K_d value for binding to PPAR γ or the concentration needed to elicit adipogenesis and insulin sensitization. Recent studies have demonstrated that activation of PPAR γ by either 15d-PGJ₂ or rosiglitazone in colonic cell lines attenuated inflammatory cytokine gene expression through inhibition of NF- κ B activation.²⁸⁹ Furthermore, both troglitazone and rosiglitazone reduced colonic inflammation in an established murine model of colitis, suggesting that PPAR γ agonists may have therapeutic potential in the treatment of inflammatory bowel disease.²⁸⁹

A recent report²³³ also proposed that PPAR γ might have proinflammatory or proatherogenic activity. PPAR γ protein is abundantly expressed in foam cells of human atherosclerotic lesions.¹³³ Foam cells are cholesterol-laden macrophages that can accumulate below the arterial wall endothelium and contribute to atherosclerotic plaque formation. The conversion of macrophages to foam cells involves the internalization of oxidized

LDL (oxLDL) particles. This internalization does not occur through the LDL receptor but rather through lipid transporters such as CD36, SR-A, and others.²⁹⁰ Treatment of HL60 monocytic leukemia cells with a combination of 15d-PGJ₂ and the RXR agonist LG100268 caused induction of macrophage markers and increased expression of CD36 and uptake of oxLDL.²³³ The induction of CD36 suggests the possibility of an oxLDL-PPAR γ -CD36 positive feedback loop.²³³ However, it is important to point out that this induction of CD36 expression required the combination of a PPAR γ agonist and an RXR agonist—treatment with a PPAR γ agonist alone could not induce CD36. Thus it is also possible that RXR agonists contribute to foam cell formation through activation of other signaling pathways.

The results presented to date portray a somewhat conflicting story on the consequences of PPAR γ activation in the context of inflammation and atherogenesis.²⁹¹ One difficulty in interpreting the results of the aforementioned experiments is that many investigators have employed the naturally occurring activator 15d-PGJ₂, which also has cellular activity independent of PPAR γ . In addition most of the data has been generated in cell culture, where results are often difficult to extrapolate to human physiology. The current clinical data in humans suggests that troglitazone-mediated activation of PPAR γ does not promote atherogenesis. Troglitazone has been shown to decrease serum PAI-1 levels in some insulin-resistant patient groups²⁹² and to reduce atherosclerosis by decreasing the intima and media thickness in carotid arteries of type 2 diabetics.²⁹³ Thus, further studies are necessary to resolve these questions and explore the potential of PPAR γ agonists in the treatment of inflammation and atherosclerosis.

PPAR γ and Cancer. Activation of PPAR γ by TZDs has been shown to trigger cell cycle arrest in logarithmically growing NIH-3T3 fibroblasts and in malignantly transformed adipogenic HIB-1B cells.²⁹⁴ The effects of PPAR γ activation on differentiation and cell cycle regulation have prompted a number of investigations into the utility of PPAR γ agonists for the treatment of cancerous tumors. Activation of PPAR γ with pioglitazone in primary human liposarcoma cells has been shown to result in differentiation and withdrawal from the cell cycle.²⁹⁵ Recently troglitazone was shown to induce terminal adipocytic differentiation in humans with advanced liposarcoma.²⁹⁶ Since current chemotherapy regimens for inoperable liposarcomas are reported to give complete response rates in less than 10% of patients, PPAR γ agonists have potential as chemotherapeutic agents for treatment of liposarcomas. PPAR γ is also expressed in human primary and metastatic breast tumors.²⁹⁷ Treatment of cultured breast cancer cells with troglitazone resulted in a reduction in growth rate and changes in cell morphology and gene expression representative of a more differentiated state. Interestingly, upon treatment with troglitazone for several days, the cells retained substantially reduced capacity for growth after cessation of drug treatment. Troglitazone has also been shown to inhibit tumor growth of MCF-7 cells in BNX triple-immunodeficient mice.²⁹⁸ The tyrosine-based PPAR γ agonist GW 7845 has been shown to significantly reduce tumor incidence, tumor size, and average tumor burden in the NMU-

induced rat model of mammary carcinoma.²⁹⁹ Additive, but not synergistic, effects were seen with GW 7845 and a suboptimal dose of tamoxifen in this model. Consistent with these effects, expression of PPAR γ is decreased in tumorigenic rodent mammary glands.³⁰⁰

PPAR γ is expressed in human colon tumors and colon cancer cell lines.^{301,302} Activation of PPAR γ in these cell lines with PPAR γ agonists caused inhibition of cell growth and cell cycle arrest, with morphologic changes consistent with colonic differentiation. Human colorectal cancer cells implanted in nude mice were shown to grow more slowly in mice treated with troglitazone, with a 50% reduction in tumor volume.³⁰¹ In addition, somatic mutations in PPAR γ have been found in a subset of human primary colorectal carcinomas.³⁰³ Each mutation led to a loss of receptor function when exposed to troglitazone or 15d-PGJ₂. These data suggest that activation of PPAR γ might have a therapeutic benefit in the treatment of colorectal cancer. However, two groups have independently reported that PPAR γ agonists increase the size and frequency of polyps in *APC^{Min}* mice, an animal model genetically predisposed to intestinal neoplasia.^{304,305} These mice are a model of the human genetic disease familial adenomatous polyposis coli (FAP), which is characterized by polyps and invasive neoplasia in the small and large intestines. In addition to FAP, the human *APC* gene is often mutated in sporadic colon cancer. It is not clear whether the increased polyp formation induced by activation of PPAR γ in this murine model resulted in invasive carcinoma, as no clear examples were documented in either study. In addition, neither study found an increase in polyps in genetically nonsusceptible mice. Therefore, the potential for increased incidence of colon tumors in humans may be confined to those at risk of developing APC-negative cancers. Due to the discrepancy in these results,^{301,303-305} however, further study of the role of PPAR γ in colon carcinogenesis is needed.

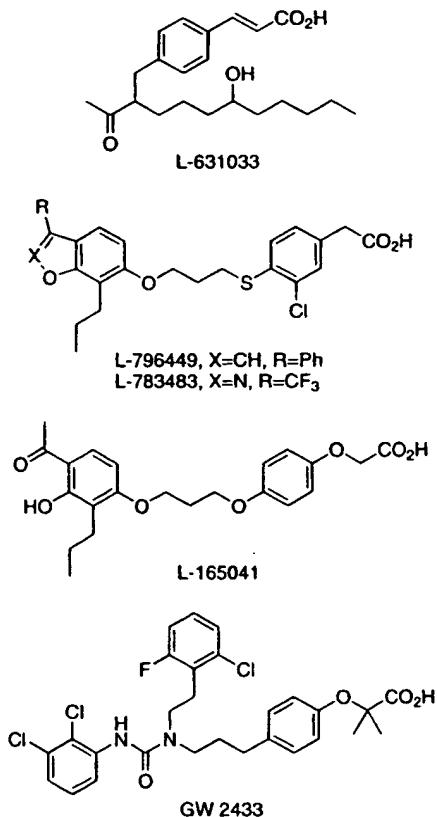
Despite the apparent dichotomy in results with colon cells, these initial studies on the effects of PPAR γ activation on cell cycle control have revealed the potential for using PPAR γ as a therapeutic target in certain forms of cancer. Although extrapolation from cell culture and rodent studies to humans is complicated and often inaccurate, the antimitotic and differentiation-promoting abilities of PPAR γ agonists warrant further human study. The fact that clinical use of troglitazone in diabetics has shown no overt toxicity in either colonic or breast tissue to date provides support for additional studies on the use of PPAR γ agonists, either alone or in combination with more conventional chemotherapeutic agents, in the treatment of human cancers.

PPAR δ

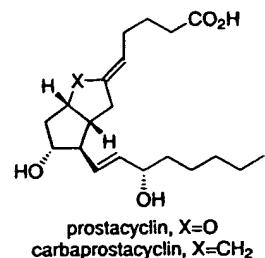
PPAR δ has been cloned from a number of species and initially given a variety of names. The receptor was first reported as PPAR β in *Xenopus laevis*⁵⁰ and NUCI in humans.³⁰⁶ Subsequently, the receptor was cloned from mice as PPAR δ ,¹⁵ NUCI,³⁰⁷ and FAAR.³⁰⁸ Today, the generally agreed upon name for this receptor is PPAR δ . The human and rodent receptors are about 90% identical in the LBD, while the frog receptor shows somewhat lower sequence identity (72%). Human PPAR δ has been mapped to chromosome 6p21.1-p21.2. PPAR δ mRNA is

ubiquitously expressed in adult rat tissues, but often at lower levels than either PPAR α or PPAR γ .⁵⁴ A study with human tissues showed that PPAR δ was present in liver, intestine, kidney, abdominal adipose, and skeletal muscle, tissues that are all involved in aspects of lipid metabolism.⁵⁵

Synthetic Ligands. Unlike the other subtypes, there are no known drugs that have been identified as working through PPAR δ .⁴⁸ Thus, part of the challenge in determining the function of PPAR δ has been the identification of potent and selective ligands for use as chemical tools.⁴⁹ It is important to note that all of the ligands published to date either have low affinity for PPAR δ or lack selectivity over the other PPARs (Table 1). The Merck group, which originally cloned NUCI, has reported a series of synthetic ligands, such as L-631033, as weak activators of the receptor.³⁰⁹ These compounds resemble fatty acids with a rigidifying ring in the middle of the chain, reminiscent of some eicosanoids. Recently this group has reported a more potent series of PPAR δ agonists.⁴⁵ The established leukotriene antagonist³¹⁰ L-165041 was identified through random screening as an activator of human PPAR δ . This compound shows 10-fold selectivity for human PPAR δ over human PPAR α and PPAR γ but is only weakly active on the murine PPAR δ with little selectivity over murine PPAR γ (Table 1). The phenylacetic acid derivatives L-796449 and L-783483 were potent dual agonists of murine PPAR δ and PPAR γ but activated all three human PPAR subtypes. We have also identified a series of potent PPAR δ ligands from a biased three-component library of ureidofibrates.⁴⁴ GW 2433 was a high-affinity ligand for human PPAR δ and a dual activator of PPAR δ and PPAR α in cell-based transactivation assays (Table 1).



Natural Ligands. In common with the other subtypes, PPAR δ is a receptor for naturally occurring fatty acids. A systematic study of the binding of fatty acids to PPAR δ found that both saturated and unsaturated fatty acids bound to the receptor.¹⁴ This binding profile was intermediate between that of PPAR α and PPAR γ . Among the polyunsaturated fatty acids, dihomo- γ -linolenic acid, arachidonic acid, and EPA bound with affinities in the low micromolar range.^{14,73} In a search for natural ligands of PPAR δ , methyl palmitate was isolated from acetone extracts of pancreatic tissue.³¹¹ The fact that the free acid was not isolated as a PPAR δ ligand is surprising, but the authors noted that the pancreas has high levels of enzymatic activity for conversion of fatty acids into their methyl esters. Another group³⁰⁸ found that palmitic acid and the metabolically stable 2-bromopalmitic acid were activators of murine PPAR δ . Several eicosanoids have been shown to activate PPAR δ including PGA₁ and PGD₂.⁷⁵ The semisynthetic prostaglandin carbaprostacyclin was reported to be one of the most efficacious activators of PPAR δ at micromolar concentrations.⁷³ Unfortunately, the naturally occurring prostacyclin (PGI₂) is too unstable to be assayed.



Protein Structure. The crystal structure of the apo-PPAR δ LBD has been determined by molecular replacement, using the PPAR γ crystal structure as a model (Figure 2B).¹⁴ The overall structure of the PPAR δ LBD was very similar to that of PPAR γ including the general size of the ligand-binding pocket. However, differences in the residues lining the ligand-binding sites result in subtle changes to the shape of the pocket (Figure 2). A second structure was determined as a cocrystal with the natural ligand EPA (Figure 6).¹⁴ Interestingly, EPA was observed bound to the receptor in two distinct conformations. The acid group and the first eight carbons adopted the same orientation in both conformations, while the tail (carbons 9–20) either pointed upward toward the upper lipophilic arm of the pocket or pointed downward into the third leg of the pocket. The ability of EPA to adopt multiple low-energy conformations within the receptor may be the molecular basis of the promiscuous binding of fatty acids to the PPARs and suggests that the need to sense multiple low-affinity hormones provided the evolutionary drive for the large ligand-binding site.¹⁴ The carboxyl group of EPA was held in place by a network of hydrogen bonds from the side chains of His⁴¹³, Tyr⁴²⁷, His²⁸⁷, and Thr²⁵³ (Figure 4B). Tyr⁴²⁷ is part of the AF-2 helix, and the hydrogen bond formed with the ligand is likely to stabilize the conformation that facilitates coactivator recruitment. The same network of hydrogen bonds was observed in the ternary complex of PPAR γ with rosiglitazone and SRC1 (Figure 4A).¹² The remarkable conservation of this

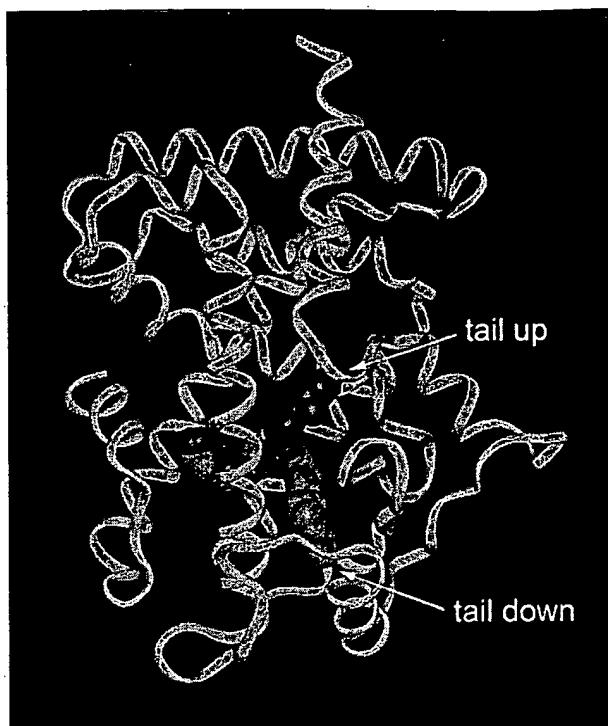


Figure 6. Crystal structure of EPA bound to the PPAR δ LBD. The PPAR δ LBD is shown as a blue worm. The two conformations of EPA observed in the crystal structure are shown in purple (tail up) and in green (tail down).

network of hydrogen bonds suggests that it must be crucial for ligand-mediated activation of these receptors. The cocrystal of PPAR δ with GW 2433 revealed that this fibrate ligand also formed the same network hydrogen-bonding interactions with the AF-2 helix.¹⁴ Interestingly, this molecule filled all the three legs of the Y-shaped pocket, which may explain why it bound with such high affinity to the receptor.

PPAR δ and Dyslipidemia. It is likely that PPAR δ is involved in lipid homeostasis because, like the other two subtypes, fatty acids and fatty acid metabolites activate the receptor. Amri showed that 3TC-C2 fibroblasts transfected with a PPAR δ expression vector became responsive to fatty acids.³⁰⁸ Specifically, two early markers of adipocyte differentiation, the adipocyte lipid-binding protein and the adipocyte fatty acid transporter (FAT/CD36), were induced. These effects may be due, in part, to PPAR δ induction of PPAR γ expression in the cells.³¹² Pharmacological activation of PPAR δ does not appear to have the same adipogenic potential as PPAR γ .³¹³ The molecular basis for these differences was recently explored. Using a series of chimeric receptors created by domain swaps between PPAR δ and PPAR γ , Castillo reported that the N-terminal domain of PPAR γ was largely responsible for adipogenic potential of the receptor.¹⁶ Thus, a chimeric receptor containing the PPAR δ LBD and DBD fused to the PPAR γ N-terminal domain could induce adipogenesis when stimulated by 2-bromopalmitic acid. These results suggest that although the highly conserved LBD and DBD are essential for the function of the receptor, it is the poorly conserved N-terminus that dictates the adipogenic potential of the receptor.

The first proposed pharmacological role for PPAR δ has been the regulation of cholesterol homeostasis.

Plasma cholesterol was raised in *db/db* mice treated with 30 mg/kg L-165041.⁴⁵ Although this compound shows only modest selectivity over PPAR γ on the murine receptors (Table 1), the pharmacological effect was ascribed to activation of PPAR δ since neither serum triglycerides nor glucose were lowered at this dose. Interestingly, in this same model a series of dual activators of PPAR δ and PPAR γ showed glucose-lowering activity, suggesting that activation of PPAR δ did not inhibit insulin sensitization through PPAR γ .⁴⁵

PPAR δ and Fertility. The role of PPAR δ has been studied in blastocyte implantation and decidualization in mice.³¹⁴ PPAR δ was the only subtype expressed in the uterus during the implantation period in mice. Expression was induced in the stroma surrounding the implanting blastocyst and became localized in the decidual zone post-implantation. This expression pattern was mirrored by the expression of prostacyclin synthase. Cyclooxygenase-2 (COX2) null mice show defects in implantation and decidualization. Since COX2 is involved in the synthesis of prostacyclin, it was hypothesized that COX2^{-/-} mice may be deficient in endogenous activators of PPAR δ .³¹⁴ In support of this hypothesis, PPAR δ activators such as carba prostacyclin or L-165041 in combination with 9-cis-retinoic acid were shown to restore implantation in the COX2^{-/-} mice. Thus, prostacyclin or its metabolites may be regulators of embryo implantation through activation of PPAR δ .

PPAR δ and Cancer. A recent report has suggested that NSAIDs mediate their antitumorigenic activity in colorectal cancer in part through PPAR δ .³¹⁵ High micromolar concentrations of the NSAIDs sulindac and indomethacin suppressed PPAR δ activity in a transactivation assay. In colon cancer cells, sulindac-induced apoptosis was partially inhibited by adenoviral overexpression of PPAR δ . The authors inferred that NSAIDs reduce the incidence of intestinal tumors by inhibiting PPAR δ function and promoting apoptosis.³¹⁵ However, this proposal has yet to be tested using more potent PPAR δ ligands.

Summary and Perspectives

It is nearly a decade since the first PPAR subtype was reported as an orphan member of the nuclear receptor gene family.⁹ Few could have predicted in 1990 that the PPARs would emerge as therapeutic targets with widespread impact in the treatment of human metabolic diseases. The initial characterization of these orphan receptors focused on their roles as lipid-regulated transcription factors and the regulation of hepatic peroxisomal enzyme expression.³¹⁶ The role of these receptors in the induction of peroxisome proliferation is now understood to be species specific, with only rodents showing this response to activators of the PPAR α subtype.⁸⁷ By contrast, all three PPARs appear to function as hormone receptors for dietary fatty acids and certain eicosanoids across multiple species and in many metabolically active tissues.³¹⁷

A full appreciation of the therapeutic potential of these receptors required the identification of synthetic ligands that were drugs with formerly unknown mechanisms of action. The fibrate and glitazone drugs were developed by a succession of pharmaceutical companies over a period of 40 years using empirical medicinal

chemistry and rodent pharmacology. Although their cellular targets were unknown, these drugs had been successfully employed in the treatment of hypertriglyceridemia or type 2 diabetes in humans. The demonstration that PPAR α and PPAR γ were the receptors through which the fibrate⁷¹ and glitazone¹⁶⁰ drugs mediate their biological activity has led to a renaissance in nuclear receptor research to develop drugs for diabetes and cardiovascular disease.^{318,319} Knowledge of the molecular targets for the fibrates and glitazones has enabled medicinal chemists to synthesize a new generation of drugs that have been optimized for activity against the human PPARs. Several of these drugs are currently in clinical development. From our own laboratories, the selective PPAR γ agonist GI 262570 is currently in phase III clinical trials for the treatment of type 2 diabetes.³²⁰ Compounds with dual PPAR γ and PPAR α activity, which may combine the benefits of insulin sensitization and lipid lowering into a single drug, are also being investigated.^{41,161,320} These dual agonists may be particularly effective in those diabetic patients that have the additional risk factor of high serum triglycerides.³²¹

Even with the rapid progress that has occurred in characterization of these orphan receptors, several intriguing questions remain unanswered. What are the natural hormones and physiological functions of the PPARs? The accumulated evidence suggests that the PPARs are receptors for fatty acids and eicosanoids. However, it is still unclear how many of the currently identified naturally occurring ligands are true physiological hormones. To qualify for this designation, it needs to be demonstrated that a candidate ligand is found at sufficient concentrations in the correct tissues and cells and that its associated biology is physiologically relevant. For example, since PPAR γ plays a critical role in regulation of lipid and glucose homeostasis through its role in adipocyte differentiation, we might expect that its natural hormone(s) would be associated with the epidemiology of type 2 diabetes.⁵ What is the pharmacology of PPAR δ ligands? While PPAR δ is likely to be another fatty acid receptor,¹⁴ its broad expression pattern gives few clues to its role in lipid metabolism or its other functions. This receptor will be a true test of the reverse endocrinology paradigm,⁴⁹ since PPAR δ does not appear to be a receptor for any class of known drugs. The results of the study of PPAR δ -selective ligands, especially in nonrodent animal models, are eagerly awaited. Do PPAR agonists have therapeutic utility beyond the metabolic disorders of diabetes and cardiovascular disease? Diets with a high content of polyunsaturated fatty acids have been claimed to show benefit in several chronic inflammatory diseases such as eczema, psoriasis, and arthritis.^{322,323} In addition, the debate on the role of dietary saturated fat as an environmental factor in the development of cancer has raged for many years. The chemoprotective effects of polyunsaturated fatty acids in animal studies are supported by human epidemiological data on the incidence of cancer in Westernized nations; however these effects have yet to be confirmed in prospective human clinical trials.^{324,325} The evaluation of synthetic PPAR agonists as drug therapies for nonmetabolic diseases will ultimately be required to answer this question.

The PPARs are now some of the most intensely studied members of the nuclear receptor gene family. Basic research on these receptors has increased our understanding of the molecular mechanism of hormone action, the role of fatty acids as hormones, and the role of adipose tissue as an endocrine organ. The promiscuity of the PPARs for their ligands and the availability of high-resolution X-ray structures has led to the development of new concepts about molecular recognition and the evolution of low-affinity hormone receptors. Throughout this research several apparent paradoxes in hormone function have come and gone, yet much remains to be explored. New technologies that enable transcript profiling through differential gene expression³²⁶ will undoubtedly increase our knowledge of the mode of action of the PPARs through the identification of their key target genes. Thus, as we enter the 21st century, we may have the potential to develop PPAR drugs with increased efficacy and safety through understanding the molecular basis of their biological action. These drugs may be particularly useful in the growing population of prediabetic patients² that exhibit multiple risk factors for cardiovascular disease, such as impaired glucose tolerance and hypertriglyceridemia.^{91,321} The success of these drugs will provide an acid test of the value of predictive molecular medicine over empirical drug discovery.

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Peter J. Brown received his Ph.D. degree from the University of Sheffield. Following postdoctoral training at Indiana University, he joined the Cardiovascular Chemistry Group at Bristol Myers. In 1989 he moved to the Glaxo Research Institute (now Glaxo Wellcome), where his current research involves the application of combinatorial chemistry to the discovery of orphan nuclear receptor ligands.

Daniel D. Sternbach received his Ph.D. degree from Brandeis University and completed his postdoctoral training at the ETH–Zurich and Harvard University. He joined the chemistry faculty at Duke University in 1979 and moved to the Glaxo Research Institute (now Glaxo Wellcome) in 1986. His research in medicinal chemistry has involved anticancer therapy and more recently metabolic diseases.

Brad R. Henke received his Ph.D. degree from the University of Illinois at Urbana–Champaign and completed his postdoctoral training at the University of California, Berkeley. He joined the Glaxo Research Institute in 1991 and is currently a Senior Research Investigator at Glaxo Wellcome. He led the chemistry team that identified the human PPAR γ agonist GI 262570. His current research interests lie in the areas of metabolic and musculoskeletal diseases.

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Review

Physiological and Therapeutic Roles of Peroxisome Proliferator-Activated Receptors

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ABSTRACT

The peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor isoforms, including PPAR γ , PPAR α , and PPAR δ , encoded by different genes. PPARs are ligand-regulated transcription factors that control gene expression by binding to specific response elements (PPREs) within promoters. PPARs bind as heterodimers with a retinoid X receptor and, upon binding agonist, interact with cofactors increasing the rate of transcription initiation. The PPARs play a critical physiological role as lipid sensors and regulators of lipid metabolism. Natural ligands for the PPARs include fatty acids and eicosanoids. More potent synthetic PPAR ligands, including the fibrates and thiazolidinediones, are effective in the treatment of dyslipidemia and diabetes. Use of selective ligands led to the discovery of additional potential roles for the PPARs in pathological states, including atherosclerosis, inflammation, and hypertension. This review provides an overview of the molecular mechanisms of PPAR action and the involvement of the PPARs in the etiology and treatment of several chronic diseases.

INTRODUCTION

THE PEROXISOME PROLIFERATOR-ACTIVATED receptors (PPARs) form a subfamily in the nuclear receptor superfamily. Three isoforms, encoded by separate genes, have been identified thus far: PPAR γ , PPAR α , and PPAR δ .

There are two PPAR γ isoforms expressed at the protein level in mouse¹ and human,² $\gamma 1$ and $\gamma 2$. They differ only in that the latter has 30 additional amino acids at its N terminus due to differential promoter usage within the same gene, and subsequent alternative RNA processing. PPAR $\gamma 2$ is expressed primarily in adi-

pose tissue,³ while PPAR $\gamma 1$ is expressed in a broad range of tissues.

Murine PPAR α was the first member of this nuclear receptor subclass to be cloned⁴; it has since been cloned from humans.⁵ PPAR α is expressed in numerous metabolically active tissues, including liver, kidney, heart, skeletal muscle, and brown fat.^{6,7} It is also present in monocytes,⁸ vascular endothelium,⁹ and vascular smooth muscle cells.¹⁰ Activation of PPAR α induces hepatic peroxisome proliferation, hepatomegaly, and hepatocarcinogenesis in rodents.⁴ These toxic effects are lost in humans, although the same compounds activate PPAR α across species.¹¹

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Human PPAR δ was cloned in the early 1990s¹² and subsequently cloned from rodents.^{13,14} PPAR δ is expressed in a wide range of tissues and cells with the highest levels of expression found in digestive tract, heart, kidney, liver, adipose, and brain.¹⁵ Thus far, no PPAR δ -specific gene targets have been identified.

As shown in Figure 1, the PPARs are ligand-dependent transcription factors that regulate target gene expression by binding to specific peroxisome proliferator response elements (PPREs) in enhancer sites of regulated genes. PPARs possess a modular structure composed of functional domains¹⁶ that include a DNA binding domain (DBD) and a ligand binding domain (LBD). The DBD specifically binds PPREs in the regulatory region of PPAR-responsive genes. The DBD, located in the C-terminal half of the receptor contains the ligand-dependent activation domain, AF-2. Each receptor binds to its PPRE as a heterodimer with a retinoid X receptor (RXR). Upon binding an agonist, the conformation of a PPAR is altered and stabilized such that a binding cleft, made up in part of the AF-2 domain, is created and recruitment of transcriptional coactivators occurs. Coactivators augment the ability of nuclear receptors to initiate the transcription process.^{17,18} The result of the agonist-induced PPAR-coactivator interaction at the PPRE is an increase in gene transcription. Downregulation of gene expression by PPARs appears, thus far, to occur through indirect mechanisms.^{19,20}

The first cloning of a PPAR (PPAR α) occurred in the course of the search for the molecular target of rodent hepatic peroxisome proliferating agents. Since then, numerous fatty acids and their derivatives including a variety of eicosanoids and prostaglandins have been shown to serve as ligands of the PPARs. Thus, these receptors may play a central role in the sensing of nutrient levels and in the modulation of their metabolism. In addition, PPARs are the primary targets of selected classes of synthetic compounds that have been used in the successful treatment of diabetes and dyslipidemia. As such, an understanding of the molecular and physiological characteristics of these receptors has become extremely important to the development and utilization of

drugs used to treat metabolic disorders. In addition, due to the great interest within the research community, a wide range of additional, putative roles for the PPARs have been discovered; PPAR α and PPAR γ may play a role in a wide range of events involving the vasculature, including atherosclerotic plaque formation and stability, thrombosis, vascular tone, and angiogenesis. New therapeutic uses for PPAR ligands may arise from these discoveries.

SYNTHETIC LIGANDS

Ligands for PPARs are summarized in Table 1. Thiazolidinediones (TZDs) were originally developed on the basis of their insulin-sensitizing effects in animal pharmacology studies. Subsequently, it was found that TZDs induced adipocyte differentiation and increased expression of adipocyte genes, including the adipocyte fatty acid-binding protein aP2.²¹⁻²³ Independently, it was discovered that PPAR γ interacted with a regulatory element of the aP2 gene that controlled its adipocyte-specific expression.²⁴ On the basis of these seminal observations, experiments were performed that determined that TZDs were PPAR γ ligands and agonists and demonstrate a definite correlation between their *in vitro* PPAR γ activities and their *in vivo* insulin-sensitizing actions.²⁵⁻²⁷

Several TZDs, including troglitazone, rosiglitazone, and pioglitazone, have insulin-sensitizing and anti-diabetic activity in humans with type 2 diabetes and impaired glucose tolerance.^{28,29} Farglitazar is a very potent non-TZD PPAR γ -selective agonist that was recently shown to have antidiabetic as well as lipid-altering efficacy in humans.²⁹ In addition to these potent PPAR γ ligands, a subset of the nonsteroidal antiinflammatory drugs (NSAIDs), including indomethacin, fenoprofen, and ibuprofen, have displayed weak PPAR γ and PPAR α activities.³¹

The fibrates, amphipathic carboxylic acids that have been proven useful in the treatment of hypertriglyceridemia, are PPAR α ligands. The prototypical member of this compound class, clofibrate, was developed prior to the identification of PPARs, using *in vivo* assays in rodents to assess lipid-lowering efficacy.³²

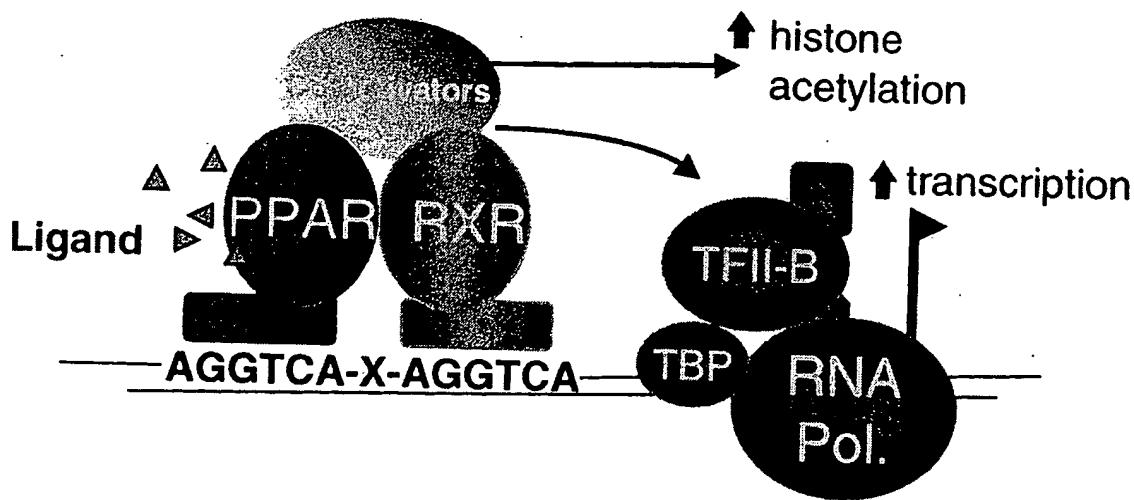


FIG. 1. Mechanism of transcriptional activation by PPAR isoforms.

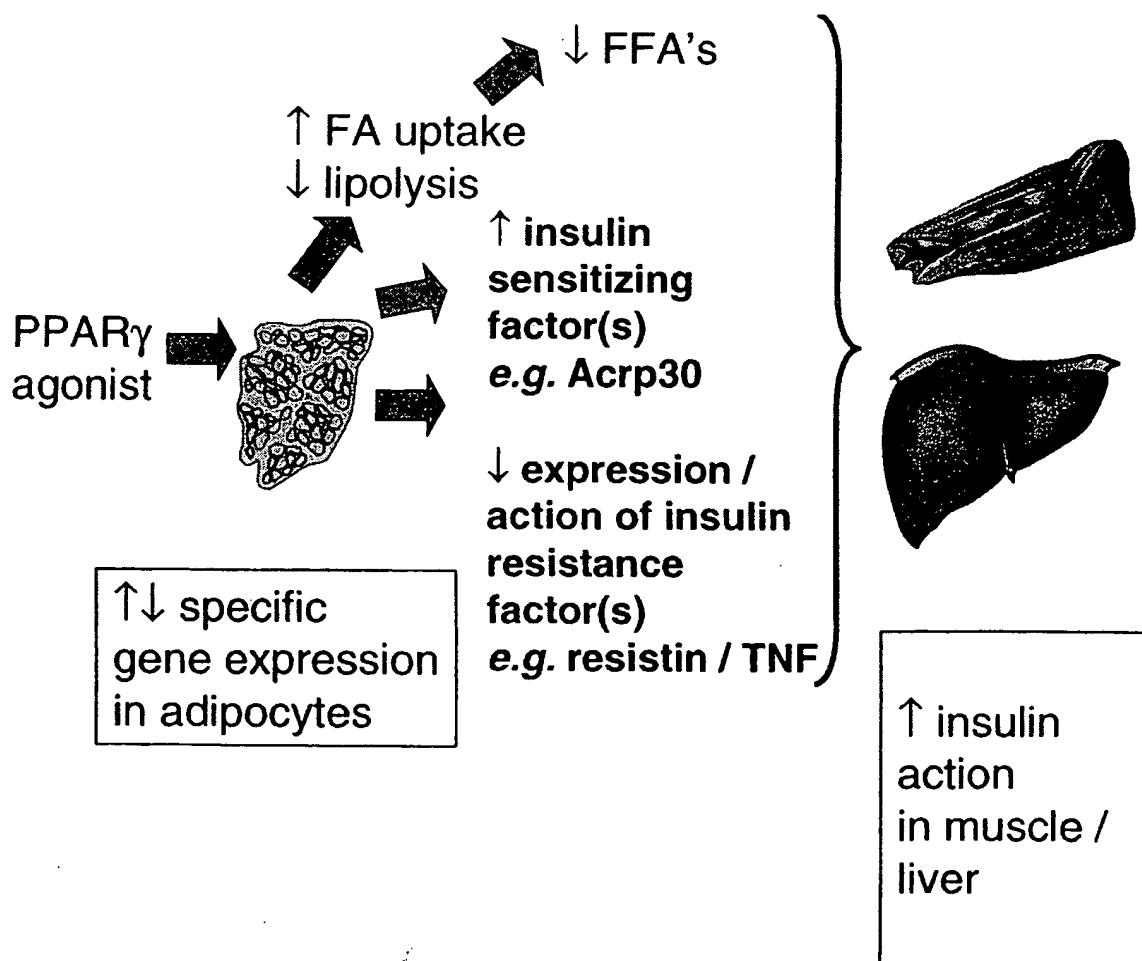


FIG. 2. Working hypothesis for PPAR γ -mediated increases in insulin sensitivity.

TABLE 1. SELECTED SYNTHETIC PPAR LIGANDS

Compounds with binding activity (IC_{50} or K_i values) to recombinant human PPAR isoforms.

Clofibrate and fenofibrate have been shown to activate PPAR α with a 10-fold selectivity over PPAR γ .²⁹ Bezafibrate acted as a pan-agonist that showed similar potency on all three PPAR isoforms. Wy-14643, the 2-arylthioacetic acid analogue of clofibrate, was a potent murine

PPAR α agonist as well as a weak PPAR γ agonist. In humans, all of the fibrates must be used at high doses (200–1,200 mg/day) to achieve efficacious lipid-lowering activity.

TZDs and non-TZDs have also been identified that are dual PPAR γ/α agonists. By virtue

of the additional PPAR α agonist activity, this class of compounds has potent lipid-altering efficacy in addition to antihyperglycemic activity in animal models of diabetes and lipid disorders. KRP-297 is an example of a TZD dual PPAR γ/α agonist³; DRF-2725³³ and AZ-242³⁴ are non-TZD dual PPAR γ/α agonists.

In order to define the physiological role of PPAR δ , efforts have been made to develop novel compounds that activate this receptor in a selective manner. Amongst the α -substituted carboxylic acids we previously described,³⁵ the potent PPAR δ ligand L-165041 demonstrated approximately 30-fold agonist selectivity for this receptor over PPAR γ ; it was inactive on murine PPAR α . This compound was found to increase high-density lipoprotein levels in rodents.³⁶ Recently, Oliver et al.³⁷ reported that GW501516 was a potent, highly-selective PPAR δ agonist that produced beneficial changes in serum lipid parameters in obese, insulin-resistant rhesus monkeys.

INSULIN RESISTANCE AND DIABETES

PPAR γ is necessary and sufficient for the differentiation of adipocytes *in vitro* and *in vivo*.³⁸⁻⁴¹ In adipocytes, PPAR γ increases the expression of numerous genes involved in lipid metabolism and lipid uptake.⁴²⁻⁴⁷ In contrast, PPAR γ down-regulates leptin, a secreted, adipocyte-selective protein that has been shown to inhibit feeding and augment catabolic lipid metabolism.^{48,49} This receptor activity might explain the increased caloric uptake and storage noted *in vivo* upon treatment with PPAR γ agonists. Clinically, TZDs, including troglitazone, rosiglitazone, and pioglitazone,^{28,29} and non-TZDs, including farglitazar,²⁹ have insulin-sensitizing and antidiabetic activity.

PPAR γ has been associated with several genes that affect insulin action. TNF α , a proinflammatory cytokine that is expressed by adipocytes, has been associated with insulin resistance.⁵⁰ PPAR γ agonists inhibited expression of TNF α in adipose tissue of obese rodents,⁵¹ and ablated the actions of TNF α in adipocytes *in vitro*.⁵² We demonstrated that PPAR γ agonists inhibit expression of 11 β -hy-

droxysteroid dehydrogenase 1 (11 β -HSD-1), the enzyme that converts cortisone to the glucocorticoid agonist cortisol, in adipocytes and adipose tissue of type 2 diabetes mouse models.²⁰ This is noteworthy since hypercortisolism exacerbates insulin resistance.^{53,54} Adipocyte Complement-Related Protein of 30 kDa (Acrp30 or adiponectin) is a secreted adipocyte-specific protein that decreases glucose, triglycerides, and free fatty acids.^{55,56} In comparison to normal human subjects, patients with type 2 diabetes have reduced plasma levels of Acrp30.⁵⁷ We found that treatment of diabetic mice and nondiabetic human subjects with PPAR γ agonists increased plasma levels of Acrp30.⁵⁸ Induction of Acrp30 by PPAR γ agonists might therefore also play a key role in the insulin-sensitizing mechanism of PPAR γ agonists in diabetes.

PPAR γ is expressed predominantly in adipose tissue. Thus, the prevailing hypothesis regarding the net *in vivo* efficacy of PPAR γ agonists involves direct actions on adipose cells with secondary effects in key insulin responsive tissues such as skeletal muscle and liver. This hypothesis is supported by the lack of glucose-lowering efficacy of rosiglitazone in a mouse model of severe insulin resistance where white adipose tissue was essentially absent.⁵⁹ Furthermore, we have shown that *in vivo* treatment of insulin resistant rats produces acute (<24 h) normalization of adipose tissue insulin action whereas insulin-mediated glucose uptake in muscle was not improved until several days after the initiation of therapy.⁶⁰ This is consistent with the fact that PPAR γ agonists can produce an increase in adipose tissue insulin action after direct *in vitro* incubation,⁶¹ whereas no such effect could be demonstrated using isolated *in vitro* incubated skeletal muscles.⁶⁰ The beneficial metabolic effects of PPAR γ agonists on muscle and liver may be mediated by their ability to (a) enhance insulin-mediated adipose tissue uptake, storage (and potentially catabolism) of free fatty acids⁶²; (b) induce the production of adipose-derived factors with potential insulin sensitizing activity (e.g., Acrp30); and/or (c) suppress the circulating levels and/or actions of insulin resistance-causing adipose-derived factors

such as TNF α or resistin.⁶³ These steps are summarized in Figure 2.

DYSЛИPIDEMLA AND ATHEROSCLEROSIS

PPAR α has been shown to play a critical role in the regulation of cellular uptake, activation, and β -oxidation of fatty acids. Activation of PPAR α induces expression of fatty acid transport proteins and enzymes in the peroxisomal β -oxidation pathway.^{44,46,64-68} Several mitochondrial enzymes involved in the energy-harvesting catabolism of fatty acids are robustly upregulated by PPAR α agonists. Peroxisome proliferators also activate expression of the CYP4As, a subclass of cytochrome P450 enzymes that catalyze the ω -hydroxylation of fatty acids, a pathway that is particularly active in the fasted and diabetic states. In sum, it is clear that PPAR α is an important lipid sensor and regulator of cellular energy-harvesting metabolism.

Atherosclerosis is a very prevalent disease in Westernized societies. In addition to a strong association with elevated LDL cholesterol, "dyslipidemia" characterized by elevated triglyceride-rich particles and low levels of HDL cholesterol is commonly associated with other aspects of a metabolic syndrome that includes obesity, insulin resistance, type 2 diabetes, and an increased risk of coronary artery disease.⁶⁹ Thus, in 8,500 men with known coronary artery disease, 38% were found to have low HDL (<35 mg/dL) and 33% had elevated triglycerides (>200 mg/dL).⁷⁰ In such patients, treatment with fibrates resulted in substantial triglyceride lowering and modest HDL-raising efficacy.⁷¹ More importantly, a recent large prospective trial showed that treatment with gemfibrozil produced a 22% reduction in cardiovascular events or death.^{71,72} Thus PPAR α agonists can effectively improve cardiovascular risk factors and have a net benefit to improve cardiovascular outcomes. In fact, fenofibrate was recently approved in the United States for treatment of type IIA and IIB hyperlipidemia. Mechanisms by which PPAR α activation cause triglyceride lowering are likely to include the effects of agonists to suppress he-

patic apo-CIII gene expression while also stimulating lipoprotein lipase gene expression.^{45,73}

Dual PPAR γ/α agonists, including KRP-297³⁰ and DRF 2725,³³ possess potent lipid-altering efficacy in addition to antihyperglycemic activity in animal models of diabetes and lipid disorders.

The presence of PPAR α and/or PPAR γ expression in vascular cell types, including macrophages, endothelial cells, and vascular smooth muscle cells, suggests that direct vascular effects might contribute to potential antiatherosclerosis efficacy.⁶⁹ PPAR α ⁷⁴ and PPAR γ ⁷⁵⁻⁷⁷ activation have been shown to inhibit cytokine-induced vascular cell adhesion and to suppress monocyte-macrophage migration. Several additional studies have also shown that PPAR γ -selective compounds have the capacity to reduce arterial lesion size and attenuate monocyte-macrophage homing to arterial lesions in animal models of atherosclerosis.⁷⁷⁻⁸¹ In addition, two recent studies have suggested that either PPAR α ⁸² or PPAR γ ^{82,83} activation in macrophages can induce the expression of a cholesterol efflux "pump" protein.

We have determined that relatively selective PPAR δ agonists produce minimal, if any, glucose- or triglyceride-lowering activity in murine models of type 2 diabetes in comparison with efficacious PPAR γ or PPAR α agonists.³⁵ Subsequently, a modest increase in HDL-cholesterol levels was detected with PPAR δ agonists in db/db mice.³⁶ Recently, Oliver et al.³⁷ reported that a potent, selective PPAR δ agonist could induce a substantial increase in HDL-cholesterol levels while reducing triglyceride levels and insulin resistance in obese rhesus monkeys.³⁷

Thus, via multifactorial mechanisms that include improvements in circulating lipids, systemic and local antiinflammatory effects, and, potentially, inhibition of vascular cell proliferation, PPAR α , PPAR γ , and, perhaps, PPAR δ agonists have a strong potential use in the treatment or prevention of atherosclerosis.

INFLAMMATION

Monocytes and macrophages are known to play an important part in the inflammatory

process through the release of inflammatory cytokines and the production of nitric oxide by inducible nitric oxide synthase. It has been demonstrated that rosiglitazone induced apoptosis of macrophages at concentrations that paralleled its affinity for PPAR γ .⁸ This ligand has also been shown to block inflammatory cytokine synthesis in colonic cell lines.⁸⁴ This latter observation offers a possible mechanistic explanation for the observed antiinflammatory actions of TZDs in rodent models of colitis.^{84,85}

Antiinflammatory actions have been described for PPAR α ligands that may be important in the maintenance of vascular health. Treatment of cytokine-activated human macrophages with PPAR α agonists induced apoptosis of the cells.⁸ Staels et al.¹⁰ reported that PPAR α agonists inhibited activation of aortic smooth muscle cells in response to inflammatory stimuli. In hyperlipidemic patients, fenofibrate treatment decreased the plasma concentrations of the inflammatory cytokine interleukin-6.¹⁰ Additional research should further investigate these provocative results and deepen our knowledge of a role for PPAR α in the physiopathology of the inflammatory process within the vasculature.

HYPERTENSION

Hypertension is a complex disorder of the cardiovascular system that has been shown to be associated with insulin resistance. Type 2 diabetes patients demonstrate a 1.5-2-fold increase in hypertension in comparison with the general population.^{86,87} Troglitazone, rosiglitazone, and pioglitazone therapy have been shown to decrease blood pressure in diabetic patients⁸⁸⁻⁹¹ as well as troglitazone therapy in obese, insulin-resistant persons.^{92,93} Since such reductions in blood pressure were shown to correlate with decreases in insulin levels,⁸⁹ they may be mediated—at least in part—by an improvement in insulin sensitivity. However, since TZDs also lowered blood pressure in one-kidney one-clip Sprague Dawley rats,⁹⁴ which are not insulin resistant, it was proposed that the hypotensive action of PPAR γ agonists is not exerted solely through their ability to improve insulin sensitivity. Other mechanisms that have

been invoked to explain the antihypertensive effects of PPAR γ agonists include their ability to (a) downregulate expression of peptides that control vascular tone such as PAI-1,^{95,96} endothelin,^{97,98} and type-c natriuretic peptide C⁹⁷; or (b) alter calcium concentrations and the calcium sensitivity of vascular cells.^{99,100} Additional research will be required in order to determine the primary mechanisms by which PPAR γ ligands lower vascular resistance.

CONCLUSION

Isoforms of the PPAR family of nuclear receptors are clearly involved in the systemic regulation of lipid metabolism and serve as "sensors" for fatty acids, prostanoid metabolites, eicosanoids and related molecules. These receptors function to regulate a broad array of genes in a coordinate fashion. Important biochemical pathways that regulate insulin action, lipid oxidation, lipid synthesis, adipocyte differentiation, peroxisome function, cell apoptosis, and inflammation can be modulated through the individual PPAR isoforms. Strong therapeutic effects of PPAR α and PPAR γ agonists to favorably influence systemic lipid levels, glucose homeostasis, and atherosclerosis risk (in the case of PPAR α activation in humans) have recently been discovered. PPAR α and PPAR γ agonists are presently used clinically to favorably alter systemic lipid levels and glucose homeostasis, respectively. Recent observations made using PPAR δ ligands suggest that this less well-characterized isoform may also be an important therapeutic target for dyslipidemia and insulin resistance, as well. Further assessment of the molecular and physiologic effects of individual PPARs will lead to a more complete understanding of the potential utilities as well as toxicities of pharmacologically modulating PPAR activities.

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